REMARKS

Claim Amendments

Claims 1 and 6 have been amended.

Claim 1 has been amended to remove the recitation "a polyanion chain of 2 to 21 negatively charged phosphate units". This amendment is not intended to limit Claim 1. Instead, the recitation of "a negatively charged phosphate residue" in Claim 1 should be interpreted to include both a single phosphate residue ***and*** a polyanion chain of negatively charged phosphate residues. Support for this interpretation is found at page 3, lines 8-11 of the specification.

Claim 1 has also been amended to recite that the method reduces non-specific binding, as opposed to non-specific binding of a target molecule. The definition of "non-specific binding" at page 12, lines 13-14, states which types of molecules can non-specifically bind to an oligonucleotide array.

Claim 6, in Step 2, has been amended to recite the activated sites are activated with one or more compounds that result in a negatively charged phosphate residue becoming bound to at least one of *i*) each of said plurality of oligonucleotides and *ii*) each of said plurality of protected regions.

No new matter has been added.

Non-Elected Subject Matter

The Examiner requests amendment of Claim 1 to remove non-elected subject matter, namely the recitation of a "polyanion chain." Applicants again refer the Examiner to MPEP § 809.04, which states that where the restriction requirement is based upon the nonallowability of a generic or linking claim, Applicants are entitled to retain claims to non-elected inventions. Applicants respectfully submit that Claim 1 is a proper linking claim for Claims 10 and 11, as discussed in detail below.

Nominally, a polyanion chain has a negatively charged phosphate residue that replaces a protecting group, as indicated by the specification at page 3, lines 8-11: "The negatively charged phosphate residue which replaces the protecting groups can be provided by reaction with a compound that covalently bonds a negatively charged phosphate residue or by attaching a polyanion chain of negatively charged phosphate residues." Thus, claims directed to a polyanion chain are properly dependent on Claim 1, because the polyanion chain provides a negatively charged phosphate group to replace a protecting group. Claim 1 should not be interpreted to

exclude instances where a negatively-charged phosphate residue is a part of a larger molecule (e.g., a polyanion).

It is recognized that the remarks in the Amendment mailed May 27, 2003 mistakenly construed the recitation "a negatively charged phosphate residue" to include only a single phosphate residue and, particularly, to exclude polyanion chains. Applicants Agent apologizes for the error.

It is noted that Applicants are not attempting to traverse the Restriction Requirement, but rather intend to correct the interpretation of the scope of Claim 1.

Rejection of Claims 1-3, 6, 7, 9 and 12 Under 35 U.S.C. § 112, Second Paragraph

Claims 1-3, 6, 7, 9 and 12 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that the instant claims require that a phosphoramidate is added as a terminal group to an existing unprotected nucleotide, but the Examiner alleges that the references previously submitted or the specification do not address such a reaction.

It is not understood why Claims 1-3, 6, 7 and 12 are included in the rejection, as Claim 9 is the only member of this group that recites phosphoramidate groups.

Claim 6, from which Claim 9 directly depends, has been amended in the second step to recite that the activated sites are reacted with one or more compounds that result in a negatively charged phosphate residue becoming bound to at least one of *i*) each of said plurality of oligonucleotides and *ii*) each of said plurality of protected regions. In Claim 9, the second step of Claim 6 is recited as ***comprising*** reacting the activated sites with certain phosphoramidates. As discussed below, a reaction sequence comprising reacting an activated group with a phosphoramidate certainly can result in binding a negatively charged phosphate residue.

Phosphoramidate chemistry is well known and understood, as demonstrated by Exhibits A, B and C. Phosphoramidite chemistry is particularly well known and understood by those who prepare nucleic acid arrays. One of ordinary skill in the art would generally know what conditions to use when performing a reaction with a phosphoramidate and would generally know what steps or conditions are necessary to obtain the desired product. Accordingly, there is no need for the subject application to describe phosphoramidite chemistry.

In the subject application, an oligonucleotide is exposed to an activator to remove a protecting group. Typically, removing the protecting group from an oligonucleotide will result in a free hydroxyl group (possibly deprotonated). The free hydroxyl group will react with a phosphoramidite as shown below:

to form a phosphite ester. R represents the oligonucleotide and R_1, R_2 and R_3 are organic moieties. It is pointed out that there is no requirement for either R_2 or R_3 to represent a nucleotide; phosphoramidate chemistry simply relies on a hydroxyl group (i.e., from an alcohol) displacing the amine group of the phosphoramidate to form a phosphite ester. If one of ordinary skill in the art was directed to prepare a negatively charged phosphate residue starting from a hydroxyl group and a phosphoramidate, he or she would immediately recognize that the initial reaction leads to the crucial phosphite ester intermediate. It is generally understood that the phosphormamidate is the key reagent in a method such as the one recited in Claim 9. (A free hydroxyl group could be reacted with other reagents, such as a phosphotriester, to form a phosphate residue.) Once the phosphite ester is formed, a skilled artisan will immediately recognize and perform the additional steps necessary to obtain the desired negatively charged phosphate residue, such that no explicit instruction is required.

It can be concluded that one of ordinary skill in the art would immediately understand how a phosphoramidate becomes a negatively charged phosphate residue. Based on MPEP § 2173.02, the instant claims are clear as written: "The test for definiteness under 35 U.S.C. 112, second paragraph is whether 'those skilled in the art would understand what is claimed when the claim is read in light of the specification.' *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 806 F.2d 1565, 1576, 1 USPQ2d 1081, 1088 (Fed. Cir. 1986)." Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claims 1-3, 6, 7, 9 and 12 Under 35 U.S.C. § 112, First Paragraph

Claims 1-3, 6, 7, 9 and 12 are rejected under 35 U.S.C. § 112, first paragraph, for not reasonably providing enablement for reducing non-specific binding of any unspecified molecule.

The Examiner states that the addition of a negatively charged phosphate group to an oligonucleotide will more likely increase non-specific binding of positively charged molecules. However, this statement does not consider the enablement of the claims as a whole, particularly as amended. Claim 1, as amended, is directed to a method for reducing non-specific binding of molecules other than a target molecule to an oligonucleotide array. Even assuming, *arguendo*, that the Examiner is correct regarding an increase in non-specific binding of positively charged molecules, non-specific binding ***as a whole*** would be reduced because of the elimination of positively charged and/or hydrophobic regions on the oligonucleotide array. Again assuming, *arguendo*, that the Examiner is correct, the only situation where non-specific binding would not be reduced is when the undesired molecules were all to have a uniform positive charge, with no hydrophobic regions or regions of negative charge. Based on the teachings of the application, this is an extremely unlikely scenario. Most non-specific binding is attributed to either proteins or insoluble salts (see page 10, lines 16-21), the vast majority of which have a hydrophobic or negatively charged surface. Thus, the claimed method clearly diminishes non-specific binding from compounds having these characteristics. For this reason, ***total non-specific binding would be decreased, even if non-specific binding of a small proportion of molecules was increased.***

It appears that the Examiner has not considered the data in Table 2, because the data regarding specular background are particularly impressive. Table 2 in Example 2 clearly demonstrates that both elimination of a protecting group and addition of negatively charged phosphate residues to an oligonucleotide array decreases specular background, which is caused by non-specific binding of insoluble metal salts. The specular background was substantially decreased in a control array when the protecting group was cleaved. The specular background was then almost completely eliminated when a number of negatively charged phosphate residues were included throughout the oligonucleotide array, and remained consistently low as the numbers of negatively charged residues increased. Thus, Example 2 indicates that both the elimination of protecting groups and the addition of negatively charged phosphate residues aid in lowering the non-specific binding of insoluble metal salts to an array in addition to a decrease in diffuse background associated with the streptavidin-phycoerthyrin complex.

The Examiner makes several statements regarding attracting positively charged molecules to an oligonucleotide array to which additional negatively charged groups have been attached. Although the presence of the additional negatively charged groups increases the overall negative charge of the array, it does not change the type of charge on the array. Oligonucleotides are

inherently negatively charged. The addition of addition negative charges to an array would not be expected to substantially affect the affinity of positively charged molecules for the array, particularly because the recited method uses phosphate groups as the source of negative charge. Thus, the presence of additional negatively charged phosphate residues in the array would not be expected to significantly increase the extent of non-specific binding by positively charged molecules. Instead, the predominant effect of the claimed method would be a decrease in non-specific binding by molecules having negatively charged or hydrophobic regions.

The Examiner appears to allege the some of the statements made in the previous Amendment could form the grounds for an obviousness rejection. Applicants respectfully disagree. The statements made in the previous Amendment are derived from the Examples and the specification as a whole. In particular, the statements are grounded in findings that: 1) photolysis of a protecting group reduced specular background, 2) additional negatively charged phosphate residues further reduced specular background and 3) additional negatively charged phosphate residues reduced diffuse background (i.e., improved the signal-to-noise ratio). Extrapolations from these findings can be made based on the known chemistry and properties, for example, of protecting groups. However, although it appears simple to interpret the Examples using conventional theory and broadly apply those findings, this can only be done using impermissible hindsight.

In summary, the instant claims ***as a whole*** are enabled. Even assuming, *arguendo*, the worst-case scenario suggested by the Examiner, non-specific binding would not be reduced by the claimed method at best only when the undesired molecules all have a uniform positive charge. This scenario is unlikely, since most molecules that would be applied to an oligonucleotide array (e.g., from a biological sample) have hydrophobic or negatively charged surfaces. Nevertheless, because the oligonucleotide arrays have a multitude of negatively charged phosphate residues to begin with, additional negatively charged phosphate residues are unlikely to have a significant effect on positively charged molecules' affinity for the oligonucleotide array. The predominant effect of the claimed method will be to reduce the affinity that molecules with hydrophobic or negatively charged surfaces have for oligonucleotide arrays. Most proteins have a hydrophobic or negatively charged surface, a property apparently shared with insoluble metal salts (see Example 2). Because proteins and insoluble metal salts are the primary sources of non-specific binding, it can be concluded that ***the claimed method***

reduces the total amount of non-specific binding to an oligonucleotide array. Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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Concord, MA 01742-9133
Dated: 12-8-03

Synthetic Organic Chemistry
Chem 131/237, Sept. 15
Lecture 5, Oligonucleotide Synthesis

DNA/RNA Structure

Pioneering Synthetic Work

Modern SPS with phosphoramidites

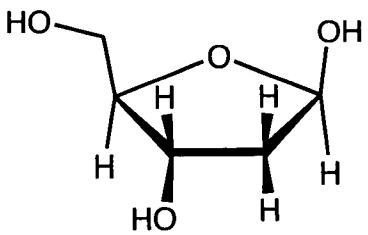
References

- Beaucage, S. L.; Iyer, R. P. Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach. *Tetrahedron* **1992**, *48*, 2223. (See also Tetrahedron Reports 329, 335)
- Bellon, L.; Wincott, F. Oligonucleotide synthesis. In *Solid Phase Synthesis*; Marcel Dekker: 270 Madison Ave, New York, NY 10016, USA, 2000; pp 475-528.
- Hayakawa, Y. Toward an ideal synthesis of oligonucleotides: Development of a novel phosphoramidite method with high capability. *Bull. Chem. Soc. Jpn.* **2001**, *74*, 1547-1565.

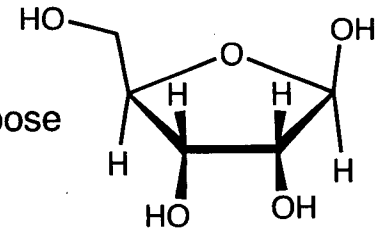
Nucleic Acids—A Refresher

Nucleic acid sugars

2-deoxy-(D) ribose

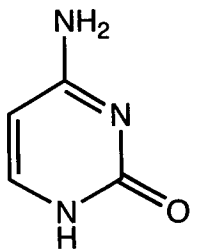


(D)-ribose

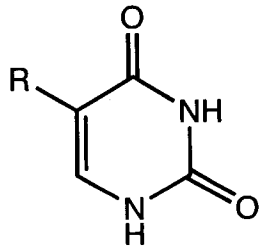


Nucleic acid bases

pyrimidines

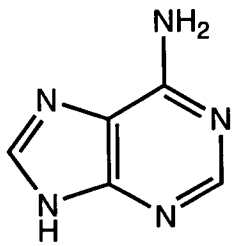


cytosine (C)

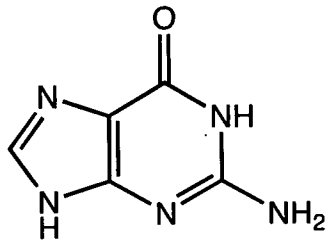


thymine (T), R = CH_3 (DNA)
uracil (U), R = H, (RNA)

purines

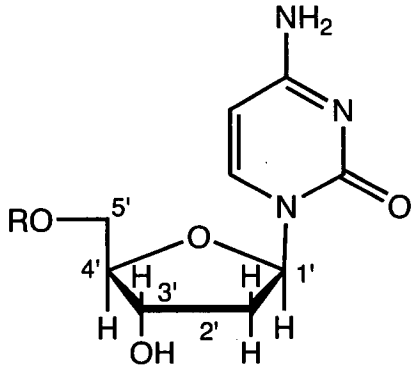


adenine (A)

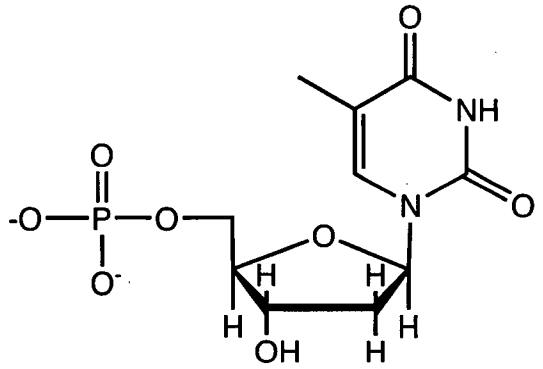


guanine (G)

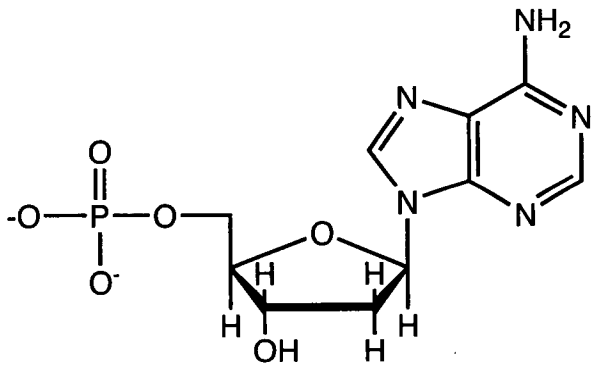
Nucleosides and Nucleotides



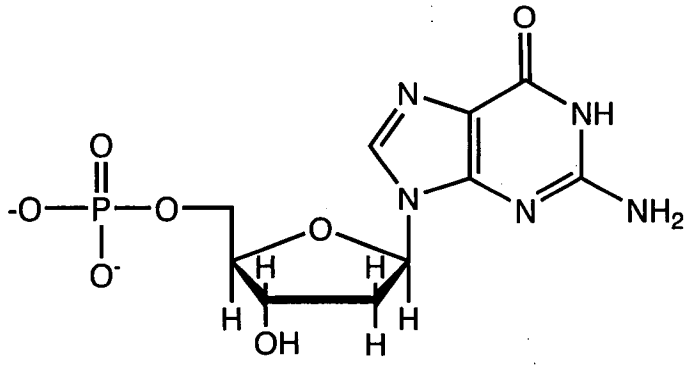
R = H, deoxycytidine, a nucleoside
R = PO_3H_2, 2'-deoxycytidylic acid, a nucleotide



(deoxythymidine)
2'-deoxythymidyic acid

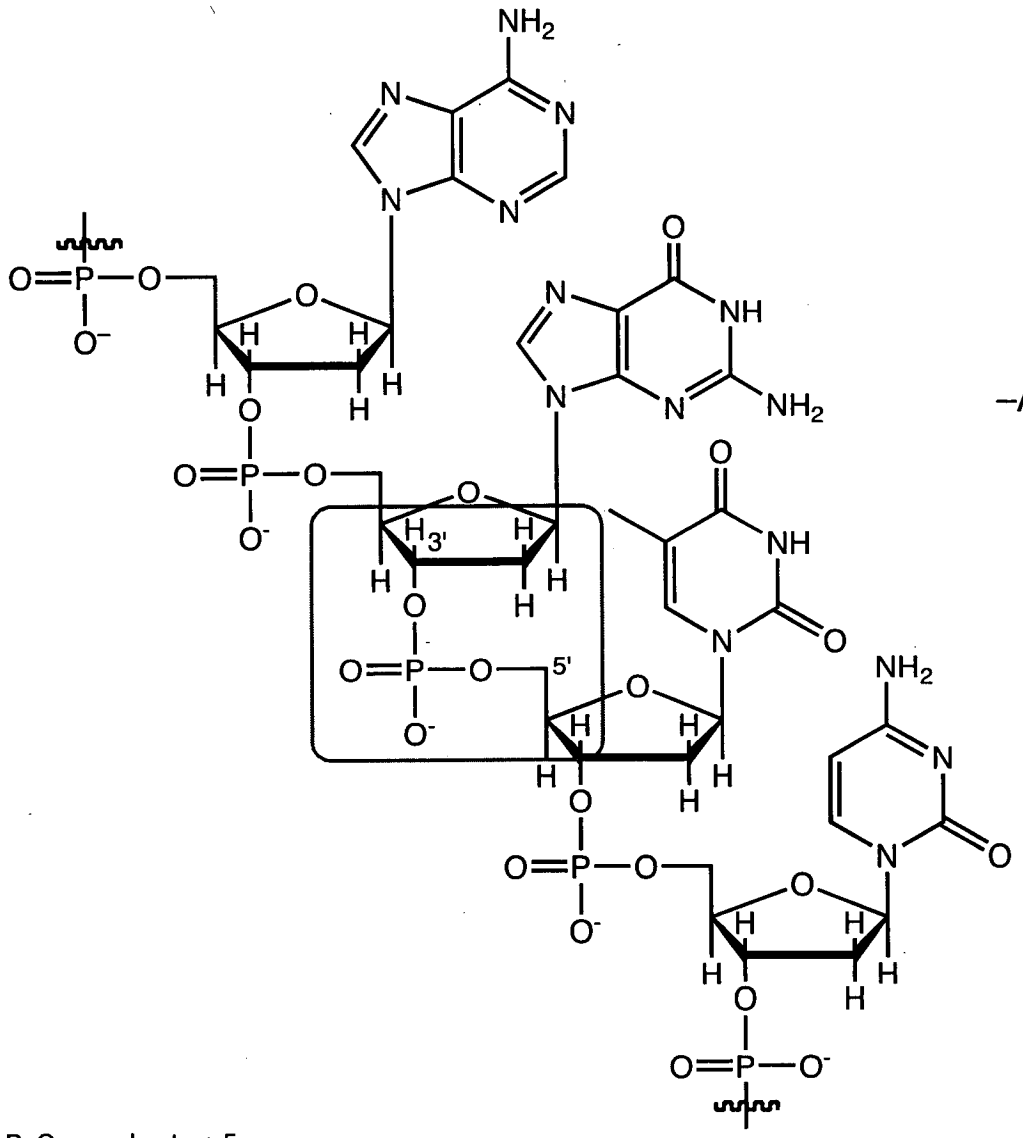


(deoxyadenosine)
2'-deoxyadenylic acid



(deoxyguanosine)
2'-deoxyguanylic acid

A Small DNA Fragment



–A–G–C–T–

Why Synthesize RNA/DNA?

- To study properties and biological effects
- For gene therapy
  - Both natural and unnatural oligonucleotides are needed
- As primers for polymerase chain reaction (PCR)
  - Amplifies genetic information
  - Protein synthesis
- Like peptides, solid phase synthesis is common
  - 10-60-mers can be made in days with automation
- Strategy is by coupling activated, protected nucleotides to make P–O bonds

Organophosphorous Compounds—Names

R_3P
phosphine
(phosphane)

$R_3P{=}O$
phosphine oxide
(phosphane oxide)

H_2POH
phophenous acid
(phosphenite)

$H_2P(=O)OH$
phosphinic acid
(phosphinate)

$HP(OH)_2$
phosphonous acid
(phosphonite)

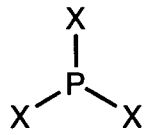
$HP(=O)(OH)_2$
phosphonic acid
(phosphonate)

$P(OH)_3$
phosphorous acid
(phosphite)

$P(=O)(OH)_3$
phosphoric acid
(phosphate)

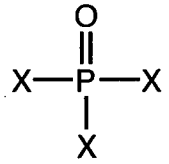
Phosphites and Phosphates

Phosphites



$(MeO)_3P$, trimethylphosphite
$(MeO_2)P(NMe_2)$, a phosphoramidate
(dimethylphosphoramidous acid dimethyl ester)
$MeOPCl_2$ methyl phosphorodichloridate

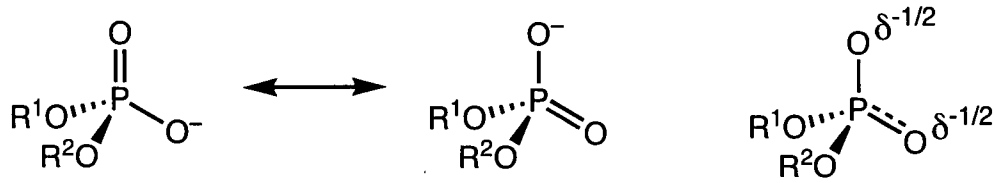
Phosphates



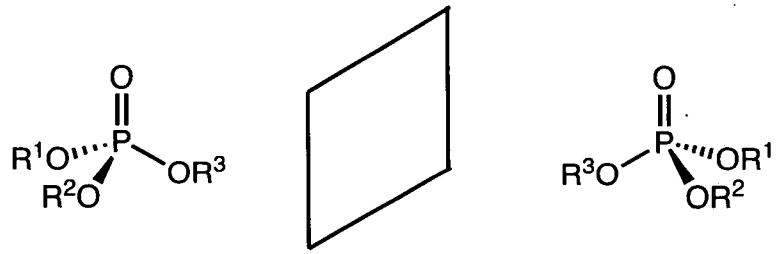
$(MeO)_3P{=}O$, trimethylphosphate
$(Me_2N)_3P{=}O$, hexamethylphosphoric triamide
$(MeO_2P(=O)Cl$, dimethylphosphorochloridate

Stereochemistry at Tetravalent P

- Diesters—P is not a stereocenter

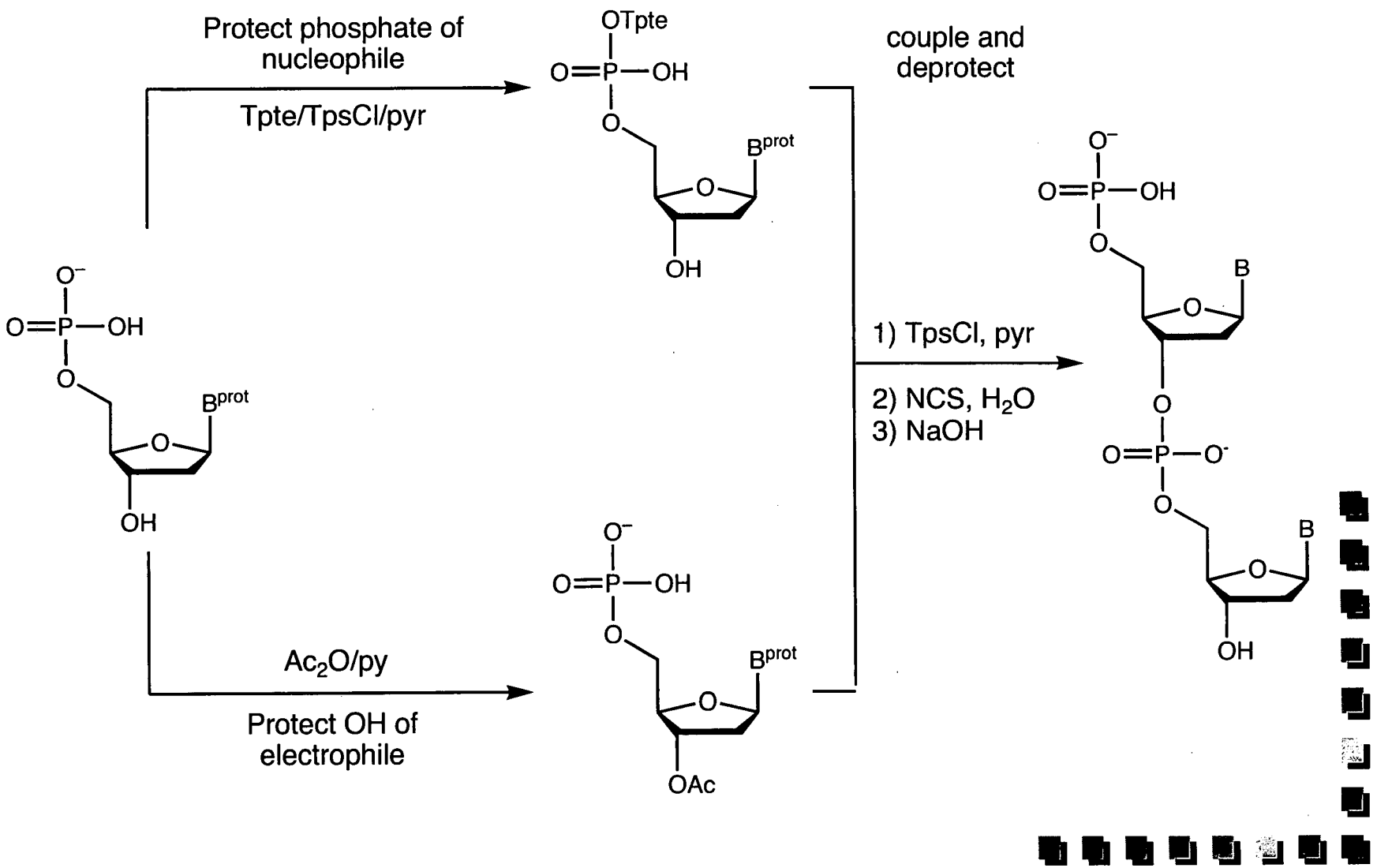


- Triesters—P is a stereocenter if $R^1 \neq R^2 \neq R^3$
  - Phosphites are also chiral, :PXYZ

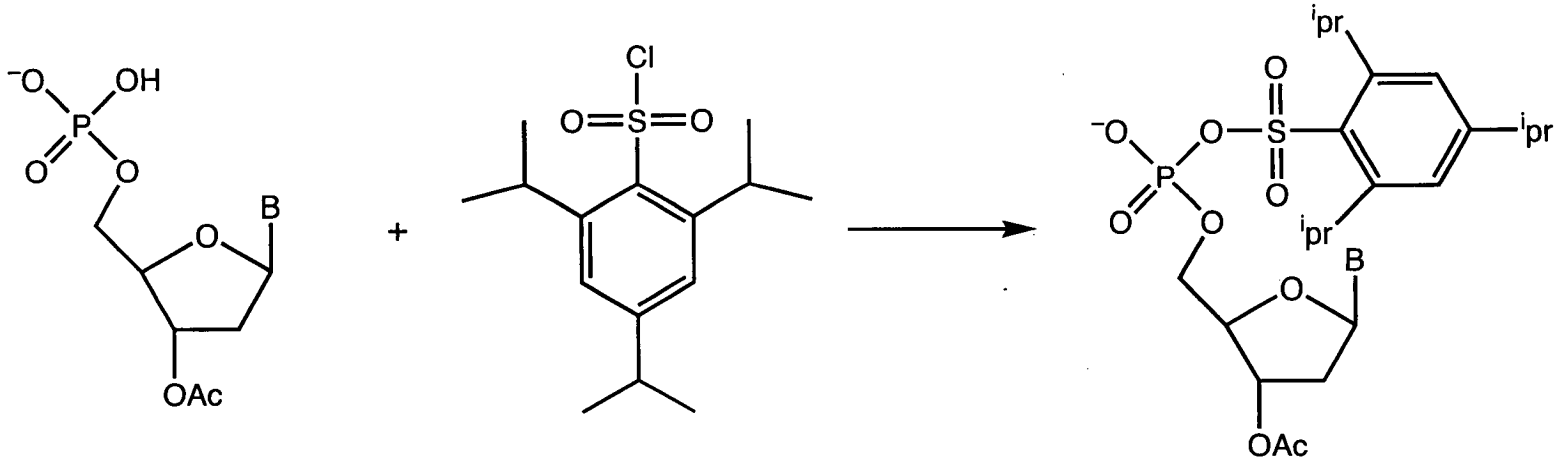


enantiomers

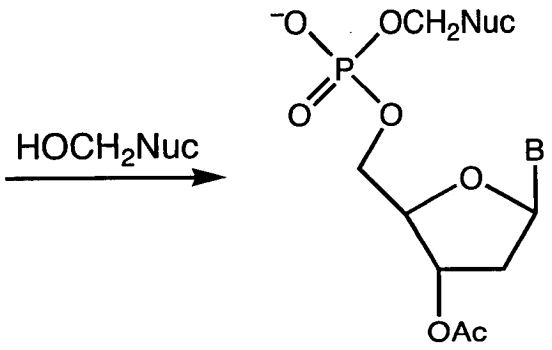
Khorana's Diester Approach



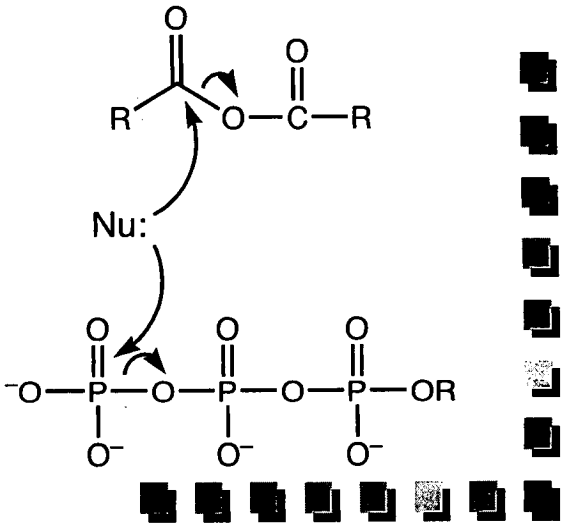
Mixed Phosphate Anhydrides



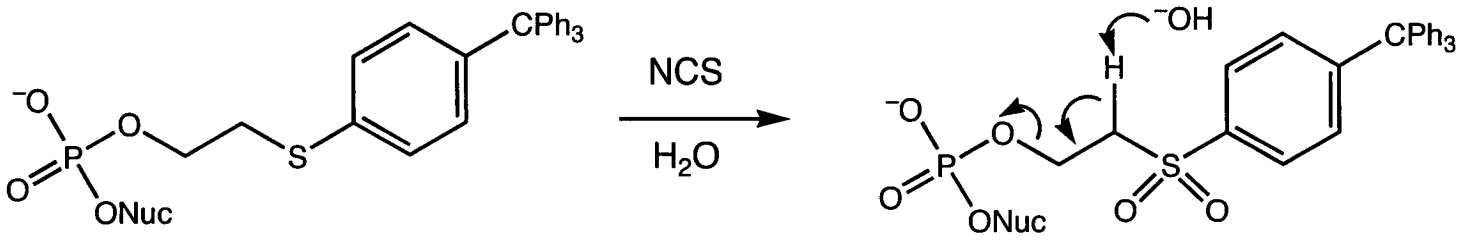
TpsCl
triisopropylbenzene
sulfonyl chloride

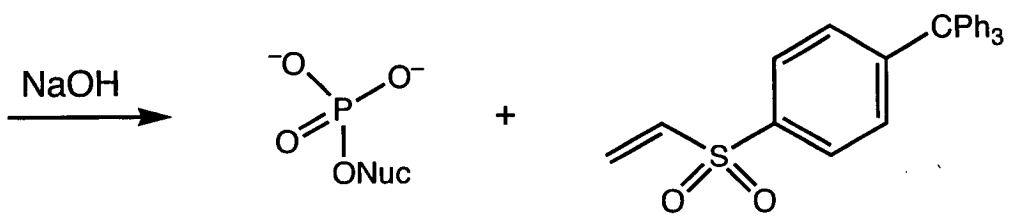


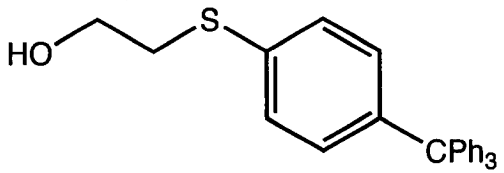
similar to



Deprotection of the Phosphate





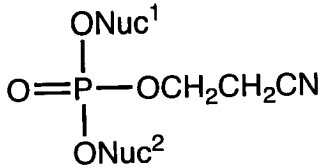


tpte, 2-(p-tritylphenylthio)ethanol

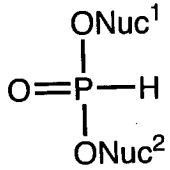
Problems with the Diester Method

- Problems:
  - Free P–O^- groups can behave as nucleophiles or be activated for displacement
  - Modest yields (~70%), slow reactions not conducive to automation
- Other methods address these problems

Phosphate Triester

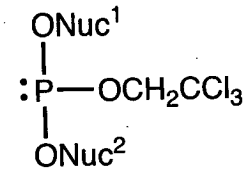


Phosphonate



oxidize with I_2/H_2O

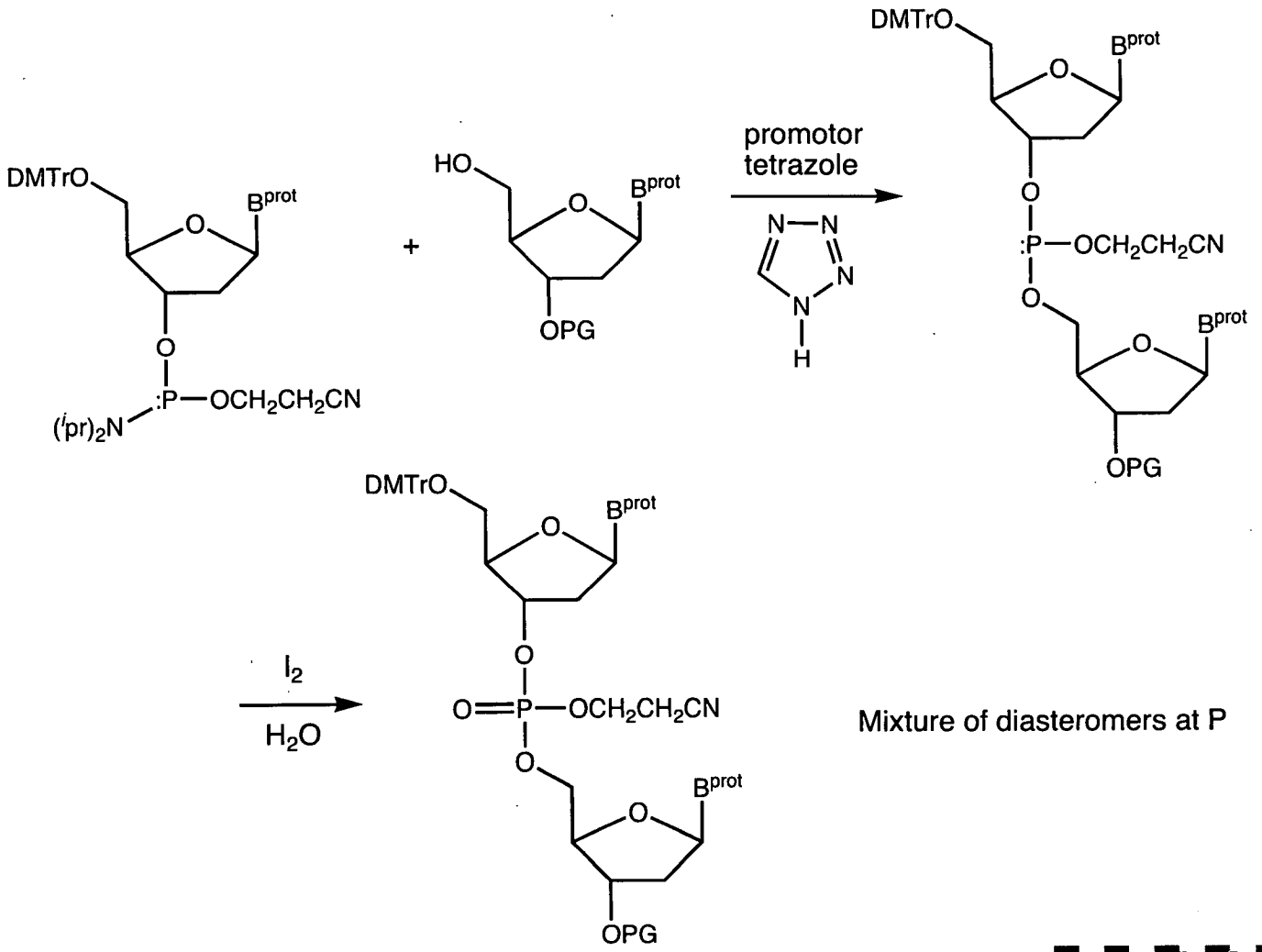
Phosphite Triester (Letsinger)



oxidize with I_2/H_2O

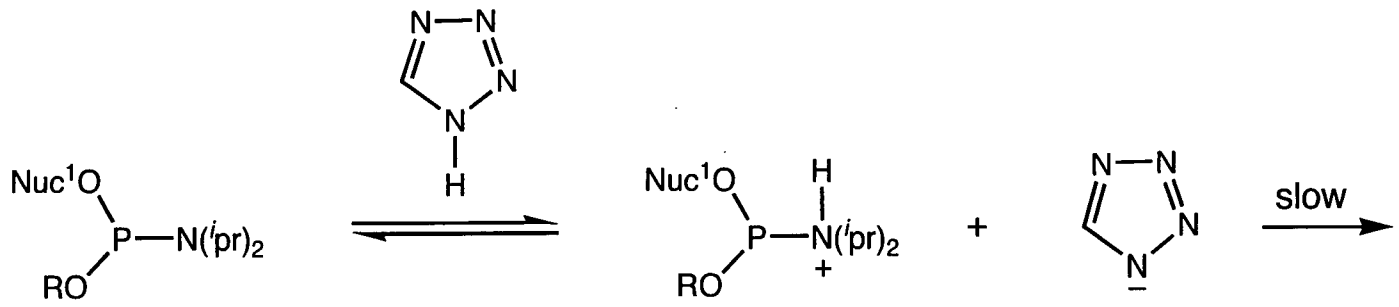
The Phosphoramidite Method

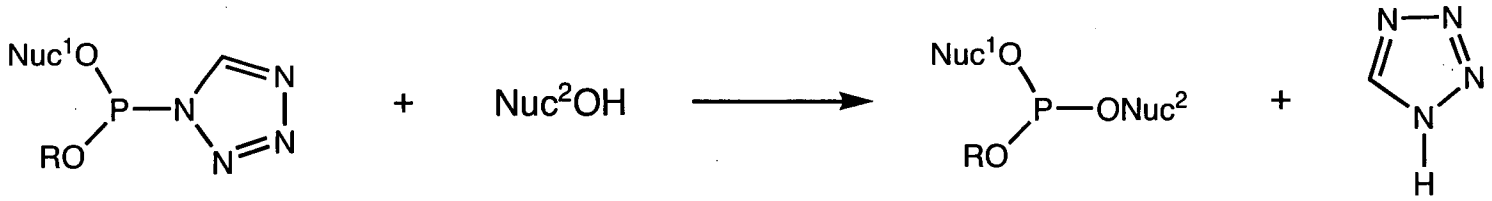
Carruthers and Beaucage



Mixture of diasteromers at P

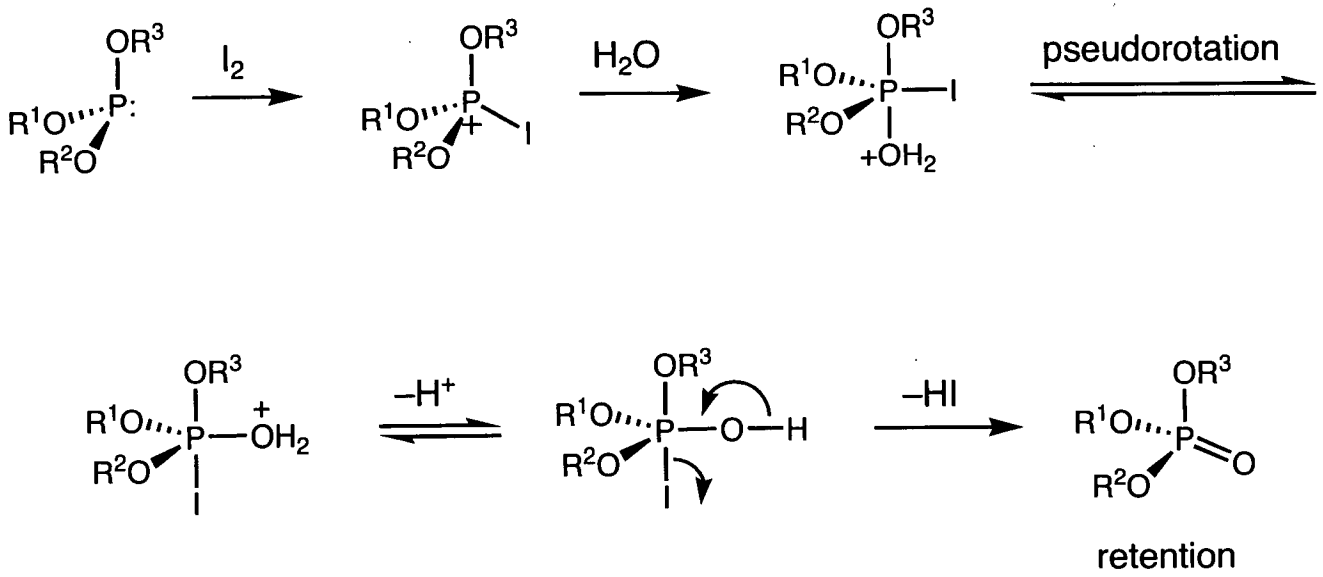
Coupling Mechanism





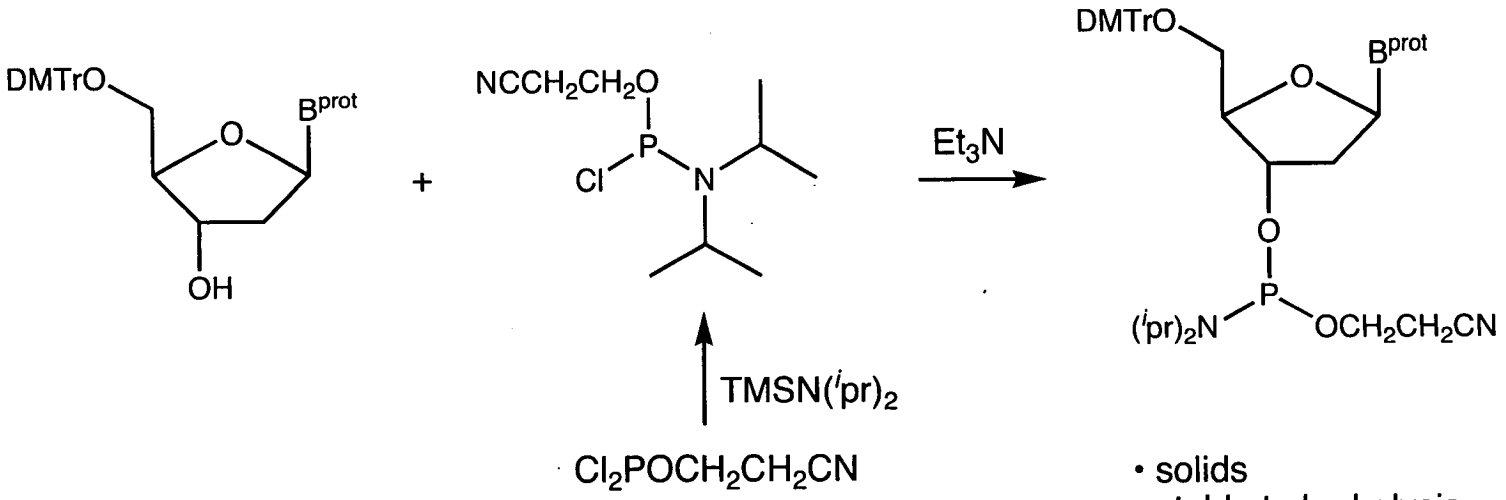
+ $HN(^{i}pr)_2$

Oxidation at P



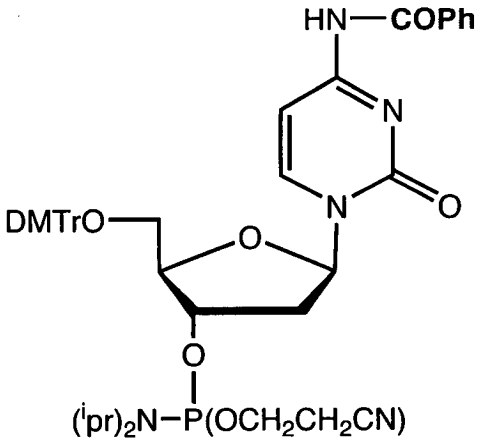
Groups add/eliminate
from apical positions on P

Phosphitylated Monomers

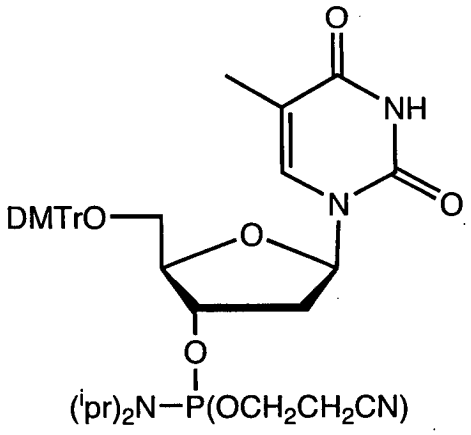


- solids
- stable to hydrolysis
- stable to storage
- readily activated for rapid coupling

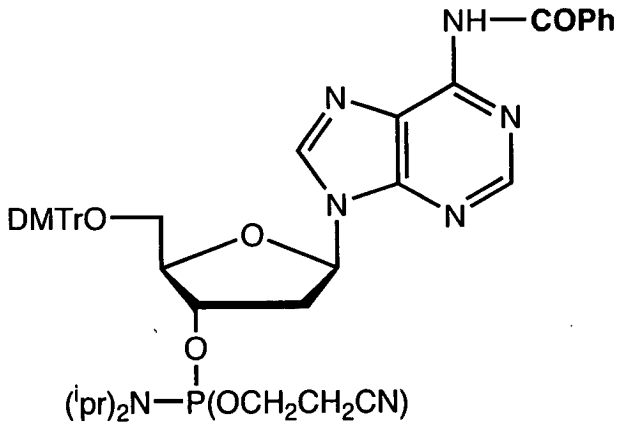
Base Protecting Groups



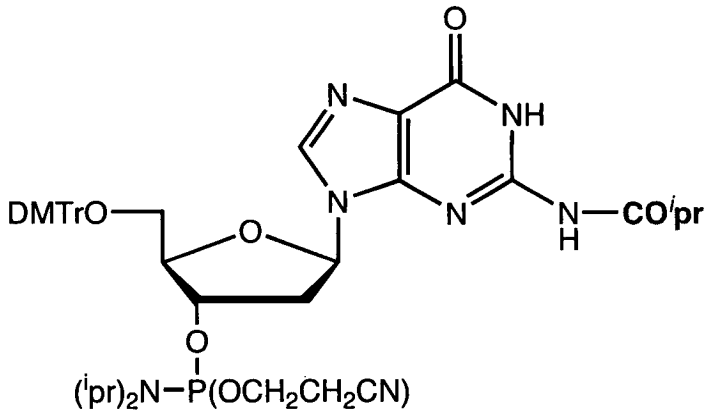
cytidine, Bz, benzoyl



thymidine, none

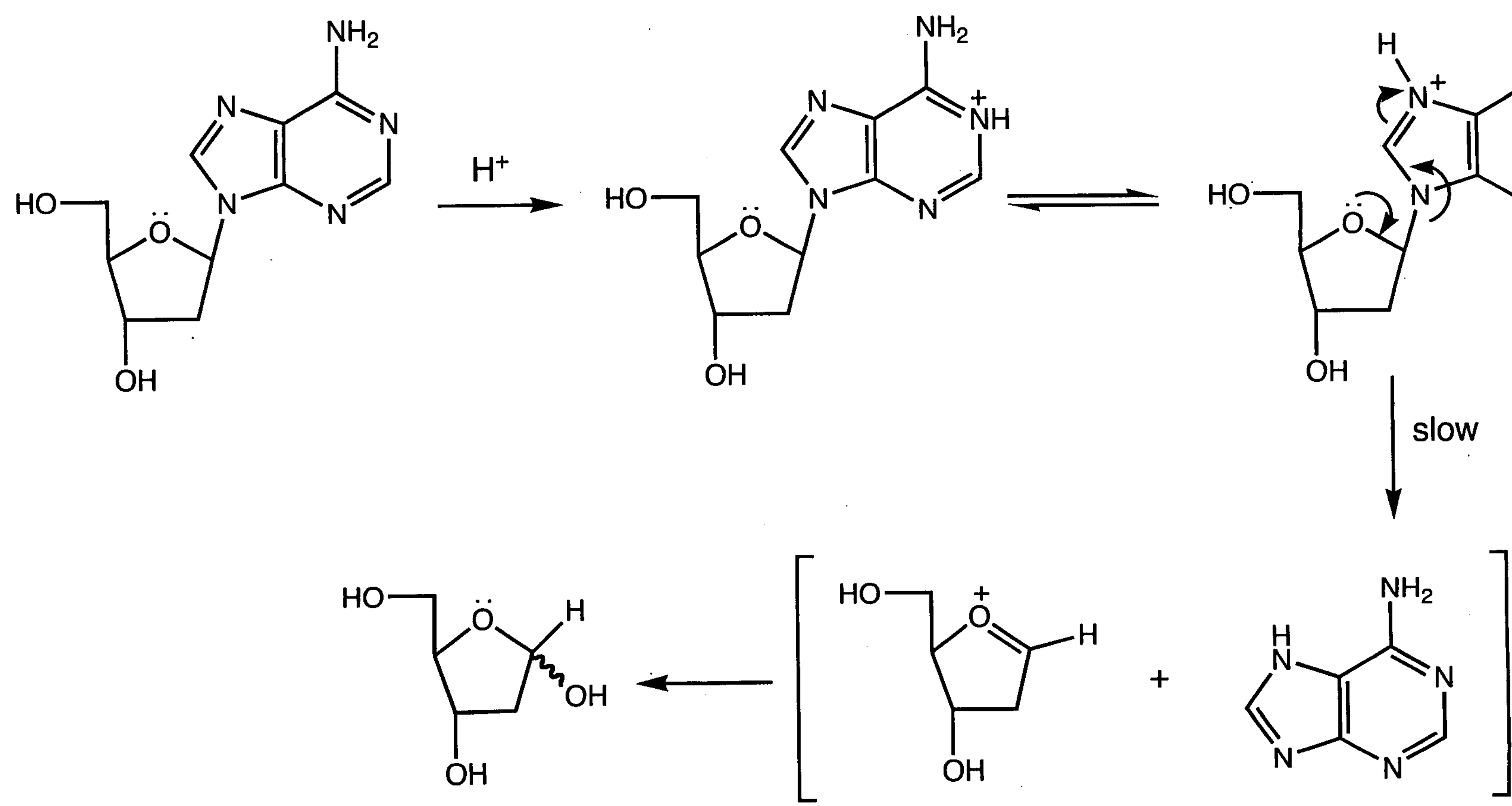


adenosine, Bz, benzoyl



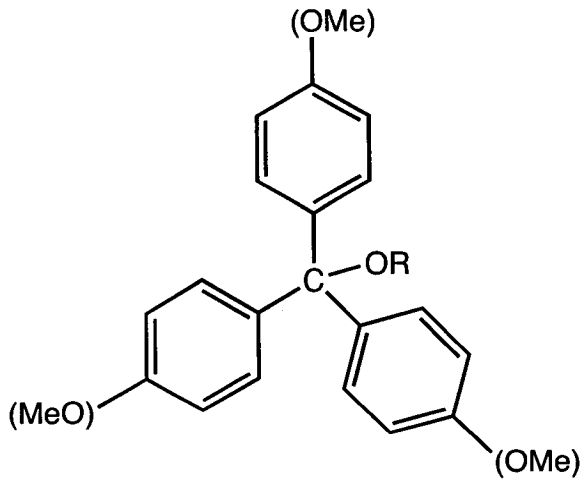
guanosine,isobutryl

Acidic Depurination



• more problematic for deoxy nucleotides
• more problematic for protected nucleotides

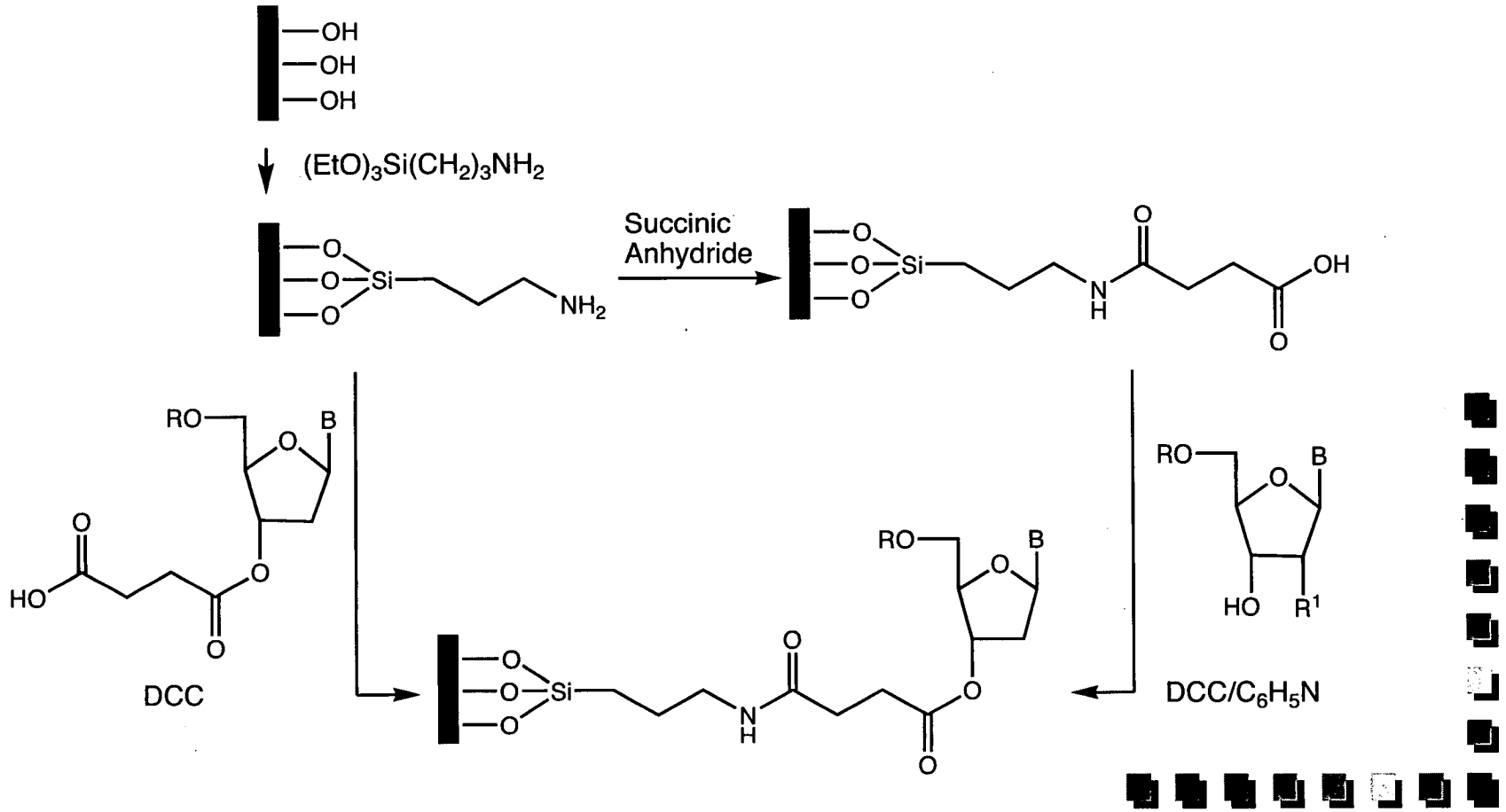
Methoxytrityl Groups



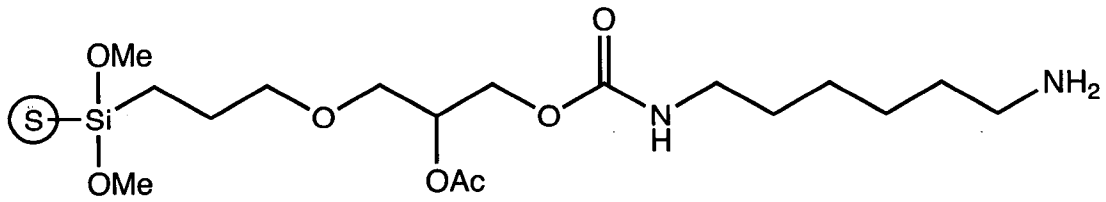
tr, trityl, too acid stable (depurination)
mtr, monomethoxytrityl, about 10X more readily cleaved
dmtr, dimethoxytrityl, cleaved with Cl_3CCO_2H or Cl_2HCCO_2H
tmtr, trimethoxytrityl, too unstable

Controlled Pore Glass (CPG)

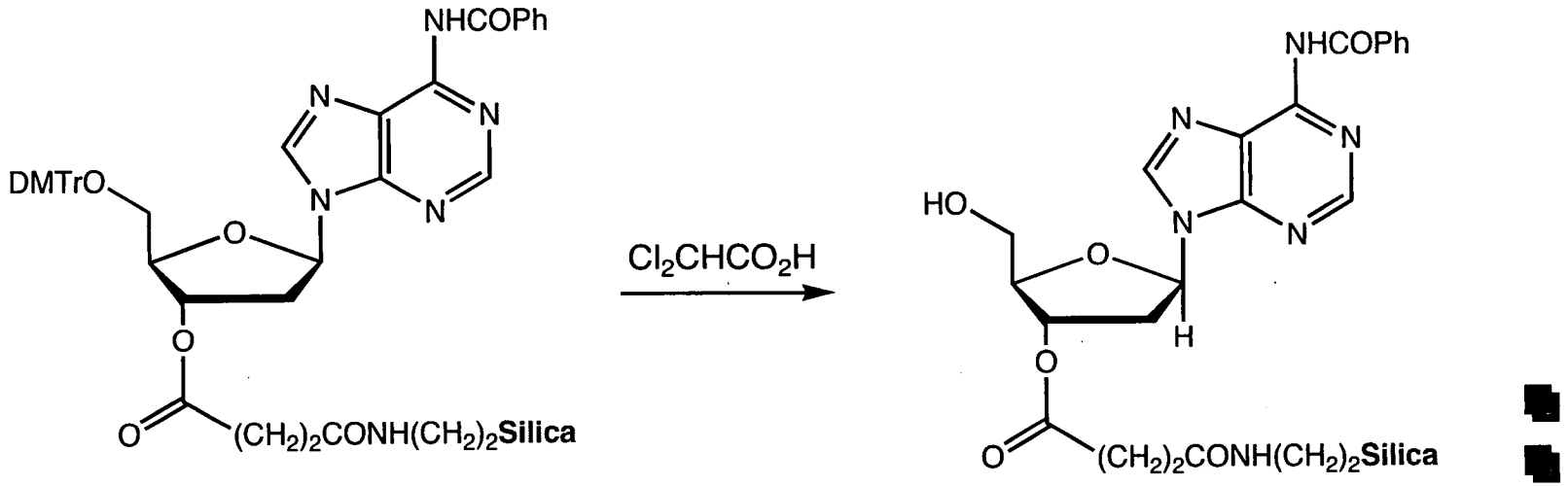
- Low loading on silica, but does not swell, reagents move rapidly through 50-100 nm pores



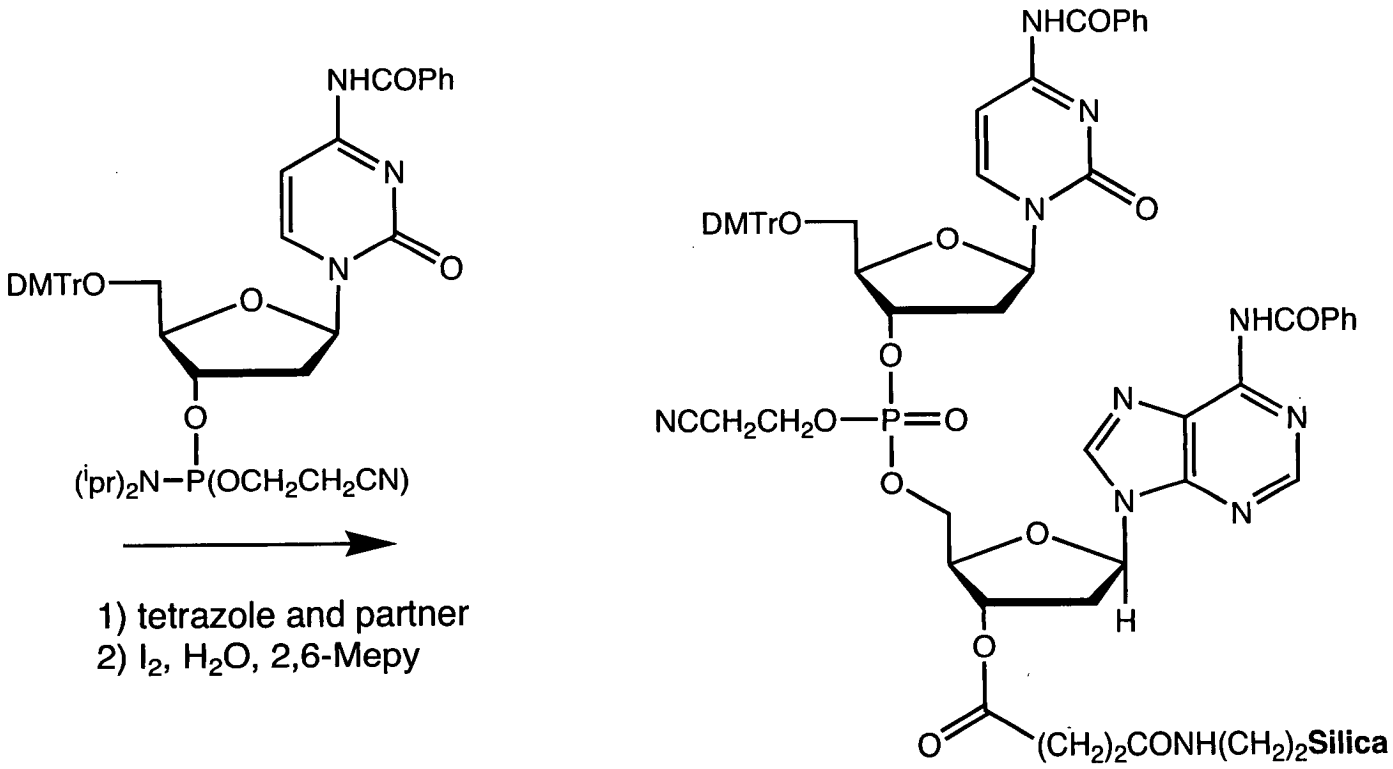
Long Chain Linkers



Oligonucleotide Synthesis



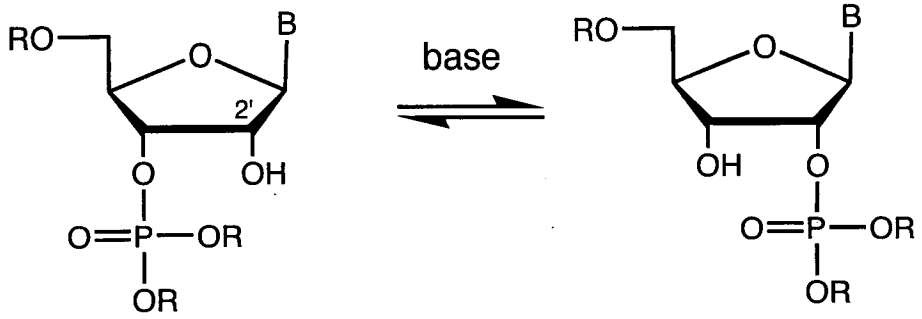
Oligonucleotide Synthesis



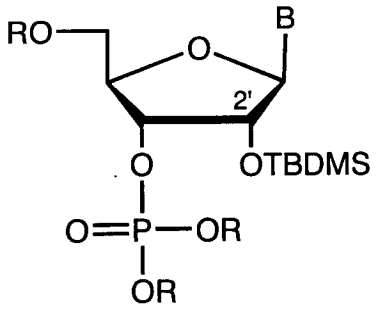
- Cycle of deprotection/coupling/oxidation
- Capping with acetic anhydride is common
- All groups removed with 9N ammonium hydroxide, then gel electrophoresis

RNA

- More delicate because of phosphate shift



- TBDMS protects 2'-OH



TBDMS (TBS) is Si(^{t}Bu)Me$_2$

Topics to be covered

5.8.02 1. Fundamentals of Solid-Phase Synthesis: Solid-Phase Peptide and Oligonucleotide Synthesis

5.10.02 2. Fundamentals of Solid-Phase Synthesis: Solid-Phase Organic Synthesis from the '60s and '70s.

5.17.02 3. Getting Attached to the Chemistry: Resins and Linkers for Solid-Phase Synthesis

5.22.02 4. Modern Solid-Phase Synthesis 1: Heterocycles, Drug Analogs, and Enzyme Inhibitors

5.24.02 5. Modern Solid-Phase Synthesis 2: Natural Products and Carbohydrates

5.29.02 6. Modern Solid-Phase Synthesis 3: Beyond Drugs and Natural Products to "Unnatural" products and "Diversity-Oriented" Synthesis

5.31.02 7. The Origins of Combinatorial Chemistry: Multiple Peptide Synthesis and Mapping Antibody Epitopes.

6.19.02 8. Combinatorial Chemistry: "Split/Mix" Libraries

6.21.02 9. Combinatorial Chemistry: Indirect vs. Direct Identification of Active Library Elements

6.26.02 10. Solution Phase Libraries: Scavenger Resins and Multi-component Condensations

6.28.02 11. Library Screening and Design

An Incomplete List of Relevant Literature Reviews

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Recommended Reading

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Neckers, D. C. (1978). Solid Phase Synthesis. Chemtech, 108 - 116

Overberger, C. G., Sannes, K. N. (1974). Polymeric Reagents in Organic Synthesis. Angewandte Chemie International Edition in English *13*, 99 - 104.

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Why You Should Care About Solid-Phase Synthesis

Even if it were the case that the only succesful solid-phase chemistries ever performed were the synthesis of oligopeptides and oligonucleotides, it would be difficult to overstate their importance. These advances created entire new areas of research, and have served as the underpinning for almost all modern biochemistry and molecular biology. Rather than elaborating, I direct your attention to the texts by Bodanszky and Gait, above.

Why You Should Care About Solid-Phase Synthesis (Continued)

Now, there are actually two other primary reasons for caring about solid-phase synthesis:

Its interesting. For me, this is aptly demonstrated by the pioneering work of several groups in the '70s, which revealed the fundamental strengths and weaknesses of solid-phase organic synthesis. (This is the "basic research" part of things, and may not appeal to everyone.)

It served as the basis for much of the early efforrts in combinatorial chemistry. As will become clear as the course progresses, a detailed understanding of solid-phase synthesis is prerequisite to appreciating or participating in many aspects of the field. While there is an increasing interest in combinatorial methods that rely entirely on solution-phase chemistry, the field really began on solid support.

Here's a flowchart illustrating the latter assertion (dates approximate):

1960's: Solid-phase peptide and oligonucleotide synthesis get started.

↓ ↓

1970's: Continued development of solid-phase peptide and oligo synthesis, including the development of effective apparati for automated synthesis.

1970's: Synthetic organic chemists begin to explore solid-phase organic synthesis. While interesting, no compelling case is made for actually bothering to do organic chemistry on solid support, and by 1980 most efforts have stagnated.

↓

1980's: Peptide chemists and biologists get interested in figuring out how to make truly huge numbers of peptides (and screen them for biological activity). This leads to the development of the first combinatorial libraries. → 1980's (late): Interest in solid-phase organic synthesis is renewed, in both academia and the pharmaceutical industry. Adaptation of "modern" synthetic reactions to the solid-phase begins.

↘ ↓

1990's: Continued improvements in the rate at which potential drug candidates can be screened (high-throughput screening) lead virtually every major pharmaceutical company to delve into the combinatorial synthesis of non-peptide, non-oligonucleotide pharmacophores.

1. *Minimized Solubility Problems.*

Ironically, carrying out chemistry on a totally insoluble polymeric support can actually circumvent solubility problems. This phenomenon was first exploited in the solid-phase synthesis of peptides, the solubility properties of which are at best unpredictable and at worst totally intractable. When constructing a short peptide on solid support, it is not necessary for the growing chain to be soluble: any solvent which effectively swells the resin and is compatible with the reagents can be used as the reaction medium. (An additional benefit is that reactions can be carried out at much higher concentrations than they might otherwise be.)

2. *Simplified Purification.*

For better or worse, you don't get to purify a substrate that's attached to solid support (at least while its still attached). This is actually a bonus, if (and only if) your solid-phase reactions are reliable and high-yielding: reaction workup consists of filtering and rinsing, without any extraction, evaporation, crystallization, etc. Similarly, if you are using an immobilized reagent, purification consists of removing product (and any residual starting material) by filtration.

3. *Improved Reaction Yields.*

To some extent, this is an extension of Item 2. The use of a large excess of one reacting partner (be it the one in solution or the one on solid support) is an excellent way to drive bimolecular reactions to completion. In solution, where both products and byproducts of a reaction are isolated simultaneously, there is a clear disincentive to use, e.g., a five-fold excess of one of the reagents. On solid support, where the excess can simply be washed away (or left behind, as the case may be), using five equivalents is as easy as using one. This, combined with the possibility of repeating a reaction cycle more than once, can lead to dramatically increased yields for a given reaction.

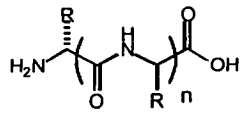
4. *Simplified Manipulation of Small Molar Quantities.*

Setting aside the cost of the resin, for a given reaction sequence, we can assume that chemical costs will vary approximately linearly with the amount of material synthesized. With oligonucleotides, for instance, where the monomeric precursors are *really* expensive, and the amounts needed for molecular biology are small (μmoles are usually more than enough), it would obviously be nice to be able to prepare μg quantities of the desired oligo. However, as any of you who have ever worked in lab know, dealing with low-mg quantities, much less μg quantities is challenging even for those with small hands and good manual dexterity. Attaching something to a solid support is a nice trick for artifically increasing the weight of the material being handled, allowing convenient manipulation up to the uiltimate cleavage step. Improvements in high-throughput screening have been such that sub-μg quantities are often more than sufficient for screening a pharmacophore, and this aspect of solid-phase synthesis is thus of increasingly broad appeal.

5. *Site Isolation.*

This is also referred to as the "hyperentropic benefit" of working on solid support. The basic idea is that, since the substrate is attached to a solid support, it ability to diffuse is dramatically restricted. This, in turn, has the potential to suppress intermolecular reactions in favor of intramolecular reactions. While its validity is taken for granted in the majority of the current literature, whether ot not this is a real effect was the subject of much contention in the '70s. (In fact, people are still arguing about it.) As you will shortly discover, it is case-dependent.

Consider your average household peptide:



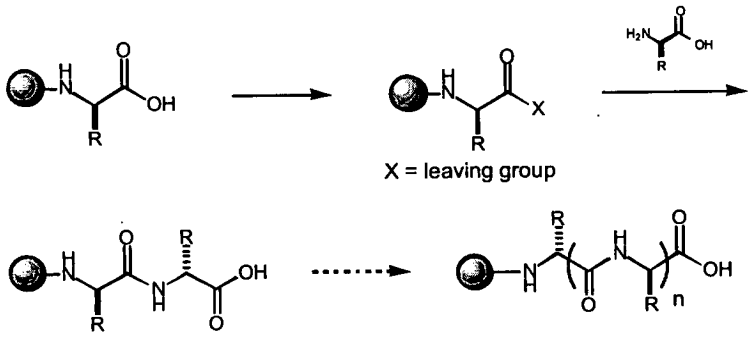
If one assumes that it is to be assembled in a stepwise manner, without enzymatic assistance, then the intrepid chemist is faced with several challenges. Among these are the experimental problems that plague solution-phase peptide synthesis:

1. Solubility.
2. Isolation.
3. Purification.
4. Racemization.

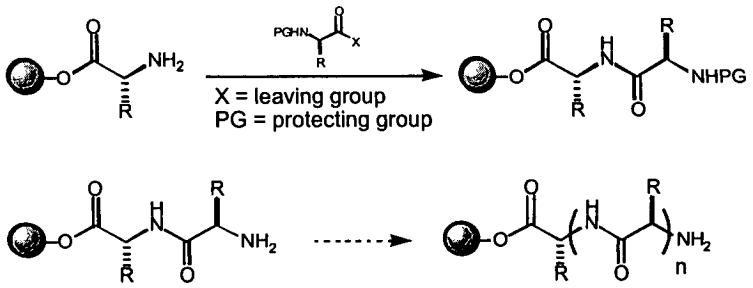
As implied previously, solid-phase synthesis is a very effective (but by no means the only) means of addressing these problems. If you'll take my word for this, then we're still left will a fundamental strategic issue: do we synthesize the peptide in the N→C direction, the way Nature does, or do we synthesize it in the C →N direction?

Here's how the two approaches look in cartoon form:

N→C Peptide Synthesis

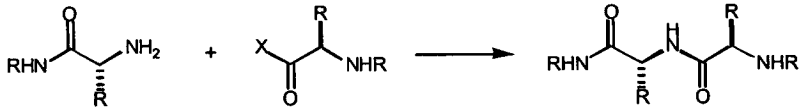


C→N Peptide Synthesis



Suppressing Racemization is a Balancing Act

As you've doubtless noted, the peptide bond-forming chemistry is the same for the two possible directions of peptide coupling:



This is where the balancing part comes in, and why we (generally) synthesize peptides in the C→N direction, even though it requires extra protecting group manipulations. It all comes down to racemization :

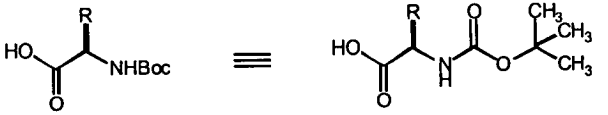
Using a more reactive activated ester (X = better leaving group) increases reaction yields, but lowers the pK_a of the α-proton, facilitating racemization.

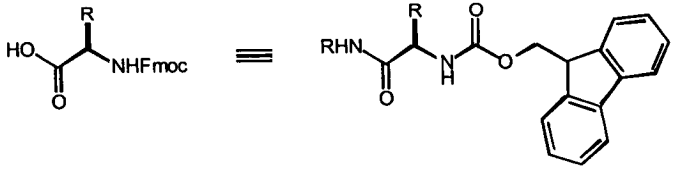
Using a less reactive activated ester decreases reaction yield, but reduces racemization. (Bear in mind that the extent of racemization will be time-dependent.)

It turns out that the best pairing of is usually the combination of a carbamate-type protecting group and a hydroxytriazole activating group

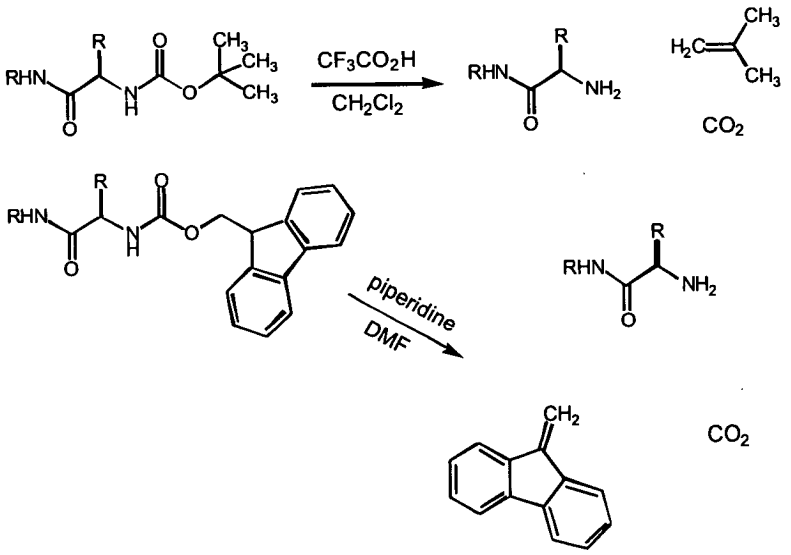
The Protecting Group Half of the Balancing Act....

It turns out, phenomenologically, that using a carbamate-type protecting group actually counterbalances the inductive effect of activated ester leaving-group (X) enough that racemization can be circumvented using these monomers. The following protected amino acids are thus ubiquitous in solid-phase peptide synthesis:



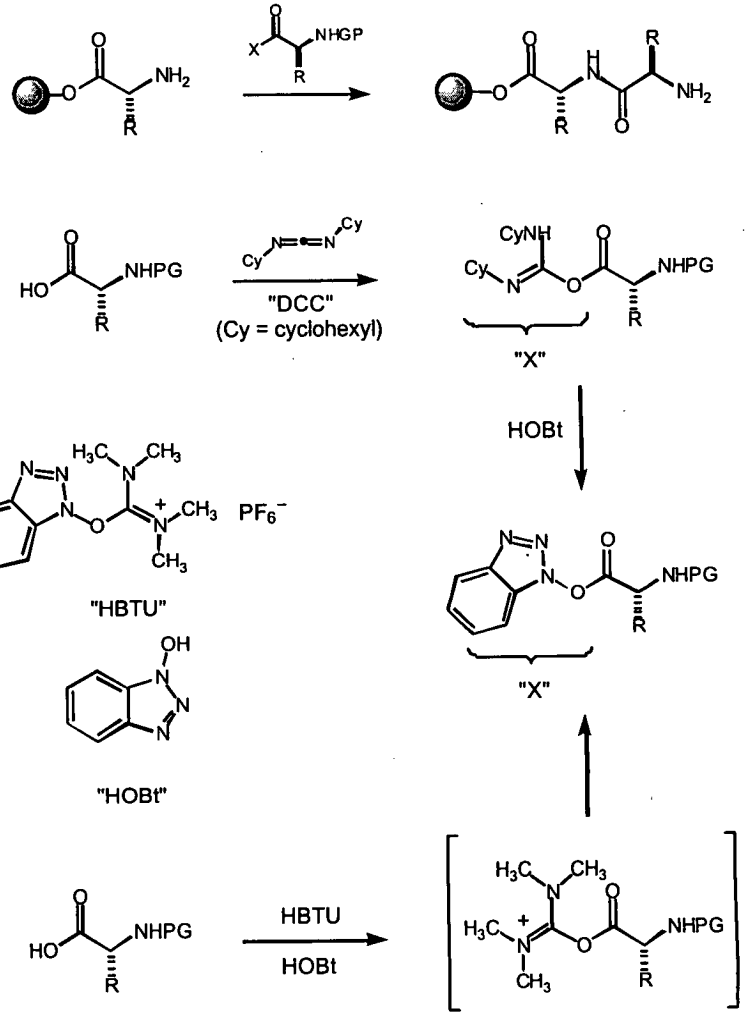


Now, just for completeness, here's how you get rid of the Boc and Fmoc groups:



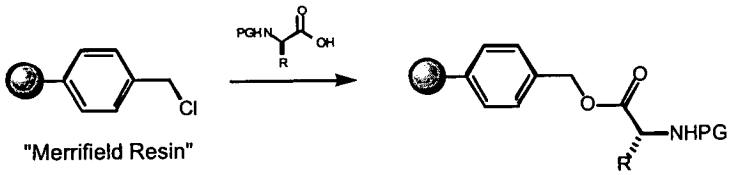
...and the Leaving Group Half

As is the case with solid-phase resins, the number of reagents available to activate a carboxylic acid for peptide coupling is enormous. For illustrative purposes, we will focus on a couple of the more common methods.



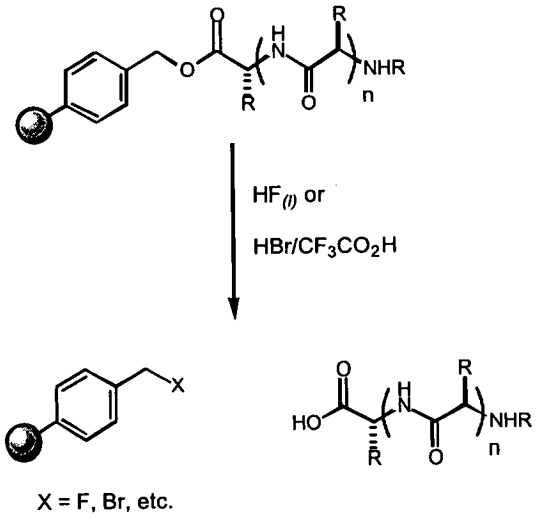
Identity of the Solid Support

At this point, we've gotten a little ahead of ourselves - you now know how to deprotect your peptides, but you don't know how to make them in the first place, or get them off the resin once made. We'll start with the resin. While there are *many* commercially available options (as you will see next lecture), most automated synthesizers use what's called Merrifield resin, which is chloromethylated polystyrene. (You can actually buy it pre-loaded with the first amino-acid residue, if you like.)



"Merrifield Resin"

Removal from the resin simply requires treatment with strong acid:



X = F, Br, etc.

Note that:

If acid-labile protecting groups are being used, they will come off during the cleavage of the peptide from the resin.

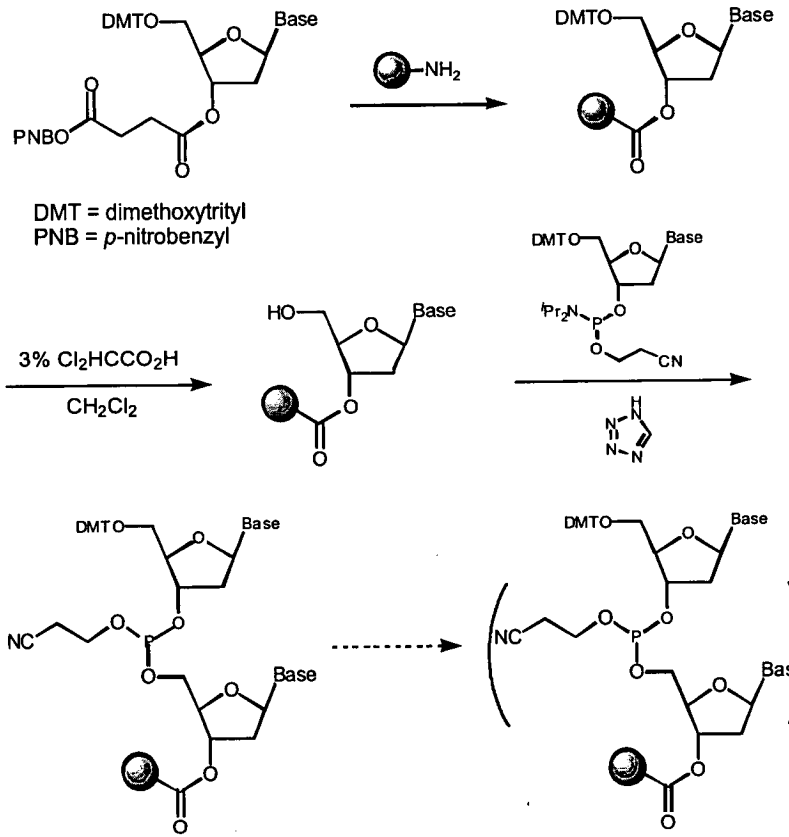
The resin can be regenerated, alhough it's rarely done.

Solid-Phase Oligonucleotide Synthesis

Much like the field of peptide synthesis, the field of oligonucleotiude synthesis is both vast and still active. For simplicity, we will address only one of the more common methods for solid-phase oligo synthesis.

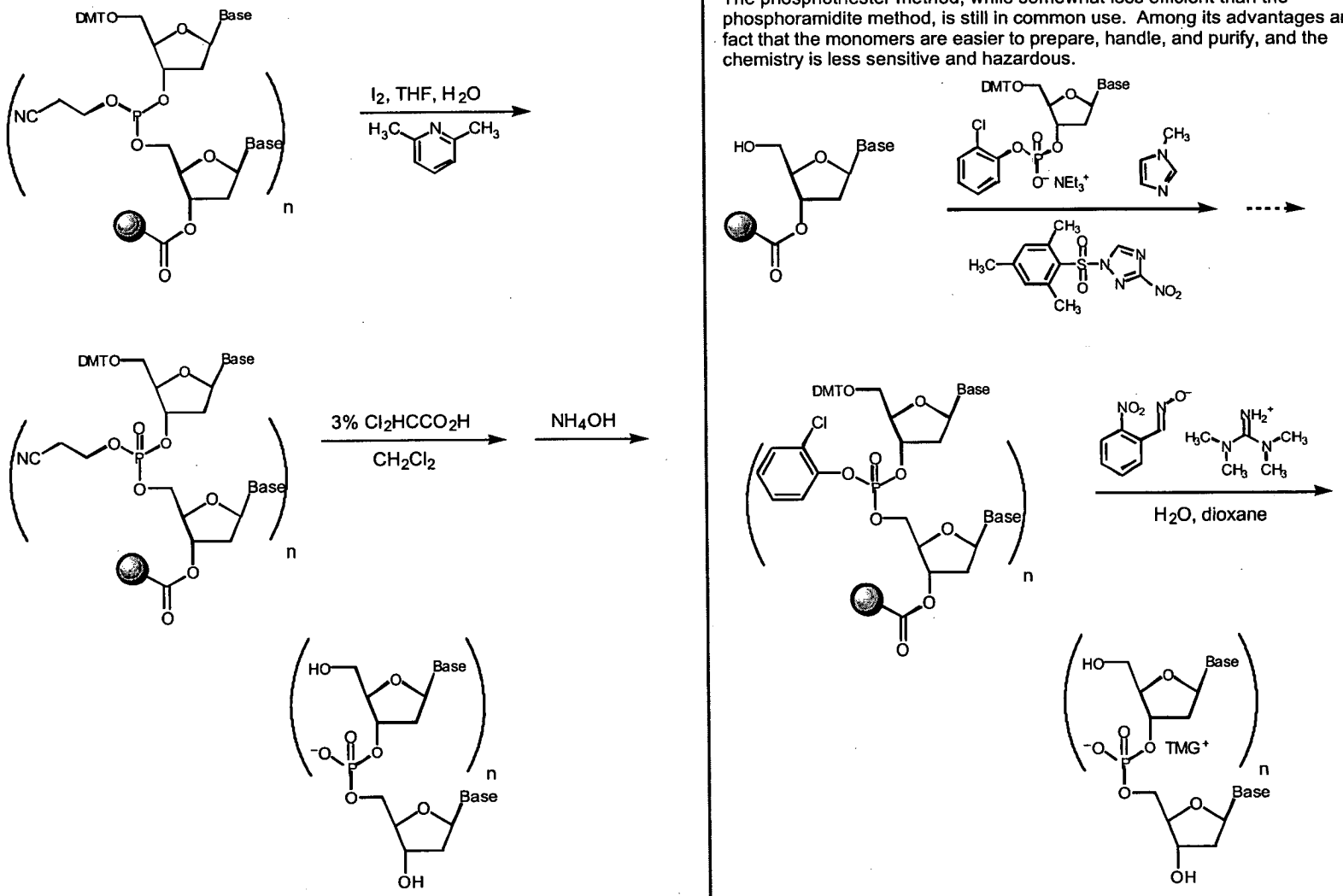
The Phosphite Triester Method

This is the most commonly used method for automated synthesis, and is often referred to as the "phosphoramidite" method. It reproducibly gives excellent coupling yields, but the phosphoramidites themselves can be a bit of a nuisance to prepare and handle.



"phosphite triester"

Oxidation and Cleavage from the Solid Support



The Phosphotriester Method

The phosphotriester method, while somewhat less efficient than the phosphoramidite method, is still in common use. Among its advantages are the fact that the monomers are easier to prepare, handle, and purify, and the chemistry is less sensitive and hazardous.

Iterative Synthesis

As mentioned earlier, one of the potential benefits of carrying out a synthesis on solid support is that an excess of reagent can be employed to drive a reaction to completion. Similarly, a reaction cycle can be repeated more than once to ensure complete conversion of a substrate. Although its somewhat non-intuitive, the empirical observation is that its often better to repeat a reaction cycle instead of increasing the amount of excess reagent used.

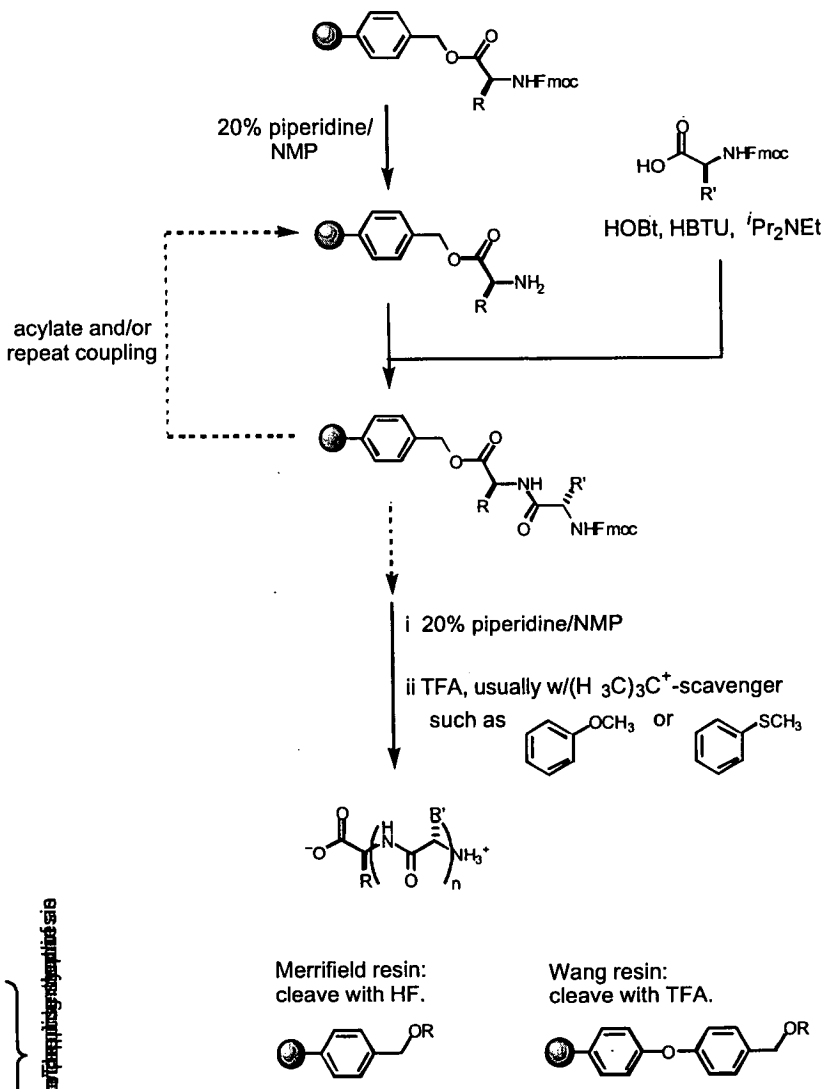
These considerations are increasingly important as the size of the polypeptide or oligonucleotide increases. Here are the cumulative yields for repeating a reaction numerous times:

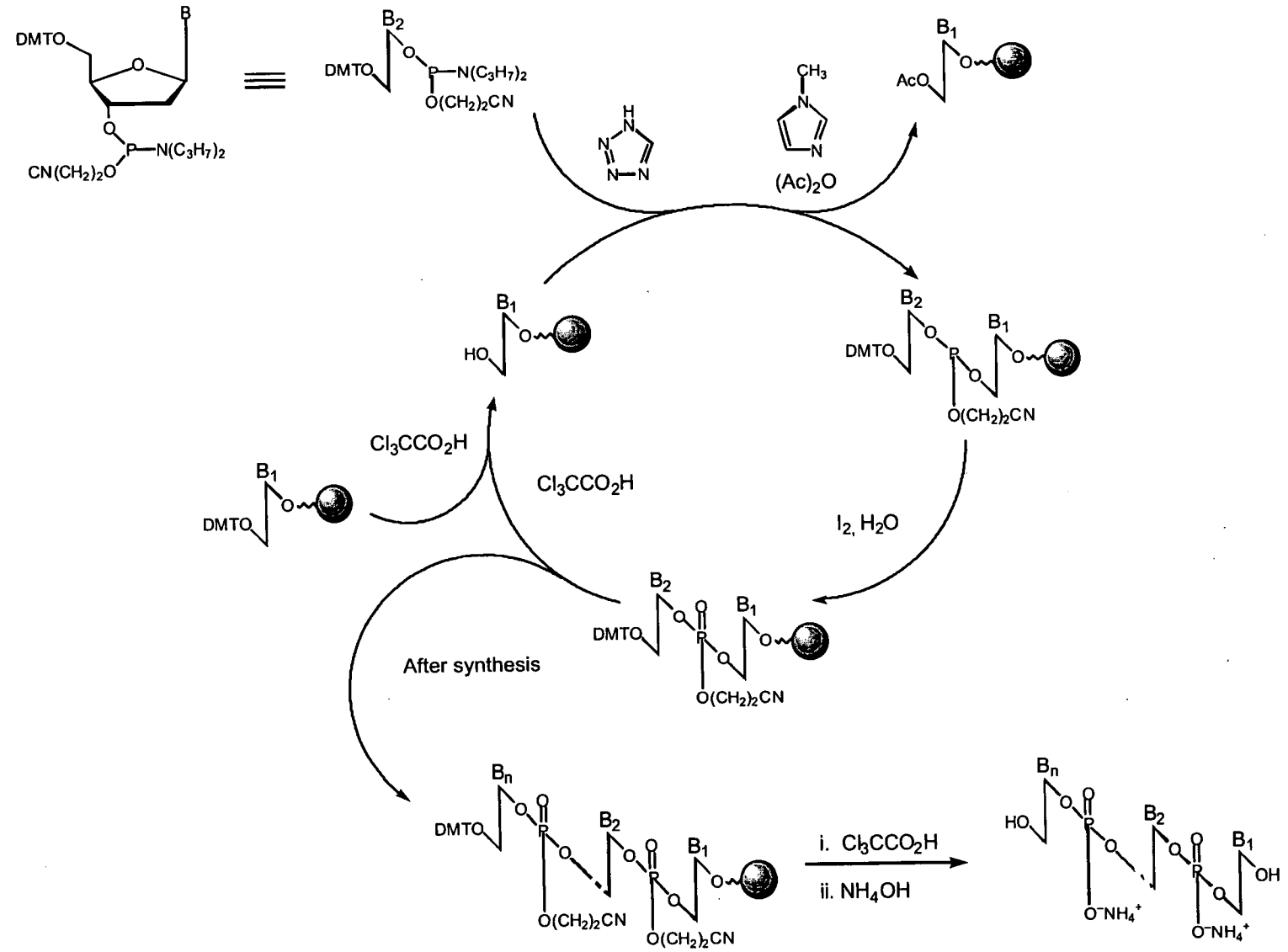
% Yield of individual step	% Yield after 5 iterations	% Yield after 10 iterations	% Yield after 15 iterations	% Yield after 20 iterations
99	97	90	86	82
95	77	60	46	36
90	59	35	21	12
85	44	20	9	4
80	32	11	4	1

This highlights one of the major differences between automated solid-phase peptide and oligo synthesis. For automated peptide synthesis (and, in fact, for manual synthesis as well), coupling steps are typically repeated more than once, in order to increase the effective yield per transformation. The above table illustrates the difference between 30 steps at 80% yield and 30 steps at 99% yield; while an overall yield of 1% might actually represent enough material for your purposes, bear in mind that you have to fish it out from the other 99%. For peptides, it is often the case that the product (n)-mer is primarily contaminated with (n-1)- and (n-2)-mers, the product of having one or two steps in (e.g.) 30 fail for a given peptide chain. Given that there are numerous possible isomers of these deletion products, and that these products are similar in length to the desired peptide, you can see that purification quickly becomes challenging.

In contrast, for solid-phase oligo synthesis, coupling steps are rarely repeated.: Instead, an acylation "capping" step is inserted in the synthetic cycle. This prematurely terminates any growing oligo chains that didn't undergo the desired coupling reaction during a given synthetic cycle. The net result is that the desired (n)-mer is easier to purify, since the impurity population is essentially evenly distributed among the (n-1)-, (n-2)-...(n-(n+1))-mers, rather than being weighted towards the impurities that most resemble the desired product.

Automated Peptide Synthesis Cycle





Thanks to Scott Singleton for the graphic.

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BIOORGANIC & MEDICINAL CHEMISTRY

Membrane-Permeant Derivatives of Mannose-1-phosphate

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Abstract—For treatment of congenital disorder of glycosylation type Ia (CDG-Ia) membrane-permeant derivatives of mannose-1-phosphate are required. Employing biologically cleavable phosphate protecting groups advantageous precursor derivatives could be synthesized following a facile approach. Their enzymatic cleavages using esterase from porcine liver (E.C. 3.1.1.1) were investigated.

Introduction

Congenital disorders of glycosylation, formerly called carbohydrate-deficient glycoprotein syndrome (CDG syndrome), belong to a new group of genetic, multisystemic, metabolic disorders. These autosomal recessive diseases were first described in 1980.[1]

The genetic defect influences the biosynthesis of N-linked glycans. CDG could be classified into two types based on the intracellular localization of the genetic defect. In type I the defect could be found in the synthesis or transfer of oligosaccharides in the endoplasmic reticulum (ER) whereas in type II the defect inhibits the processing of the N-linked glycoproteins in the Golgi.

In CDG-Ia phosphomannomutase 2 (PMM 2) is deficient, therefore the conversion of mannose-6-phosphate to mannose-1-phosphate fails, and thus the supply of GDP-mannose will be limited. Because GDP-mannose is the decisive mannose donor the biosynthesis of glycoproteins is significantly disturbed. One way of dealing with the PMM 2 deficiency would be treatment of patients with mannose-1-phosphate from external sources.

Due to the high polarity, mannose-1-phosphate is unable to penetrate through cellular membranes.[2,3] To overcome this limitation biologically reversible protecting groups previously used for carboxylic acids, phosphonates, nucleotides and inositoles[4–8] could be employed to form neutral phosphotriester. Suitable for this purpose seem to be the acetoxymethyl (AM) and the pivaloyloxymethyl (POM) esters. After penetration the acyloxy phosphate ester is expected to be converted into the parent compound by intracellular enzymatic cleavage by carboxylate esterases.[9,10] The acyloxy ester linkages should be hydrolyzed to the hydroxymethyl analogues. This intermediate in turn is chemically labile and looses one mole of formaldehyde[11,12] (Scheme 1). In order to test this in vitro, initial enzymatic deprotection experiments were conducted. For improving the lipophilicity the hydroxy groups of the mannose moiety were masked as different esters and carbonates.

Results and Discussion

Synthesis

Starting from mannose the benzyl mannopyranoside was obtained by Fischer glycosylation with benzyl alcohol.[13] This compound was converted into the appropriate substituted mannopyranosides using butyryl chloride, pivaloyl chloride or *iso*-propyl chloroformiate.[14,15] Subsequent hydrogenolysis of the benzyl groups on Pd/C (10%) yielded in the anomerically unblocked mannose derivatives **1–3**. Further reaction with dibenzyl di-*iso*-propylphosphoramidite using 1H-tetrazole gave the phosphite triesters which were oxidised in situ by *meta*-chloroperbenzoic acid (MCPBA) to the appropriate phosphate derivatives[16] **4–6**. Subsequently the benzyl groups were removed by hydrogenolysis on Pd/C (10%). The resulting phosphates **7–9** were converted into their acetoxymethyl (AM) and pivaloyloxymethyl (POM) esters **10–15** employing bromomethylacetate or iodomethylpivaloate,

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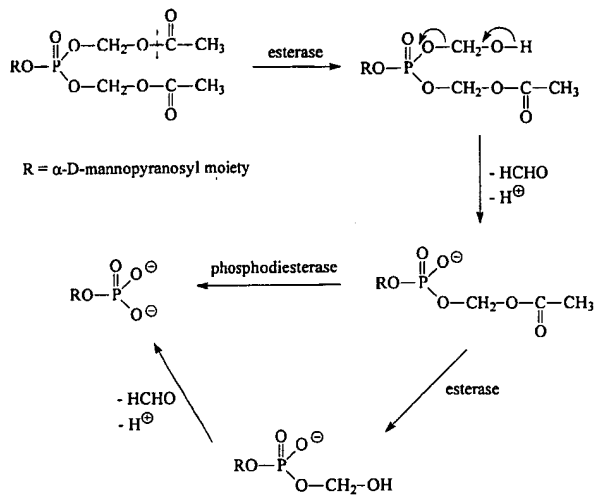


Scheme 1.

respectively, in the presence of *N*-ethyl-di-*iso*-propylamine (DIPEA) (Scheme 2).

The poor nucleophilicity of the phosphate and the lack of stability of the phosphate group obvious by the anomerically free mannose byproduct could be the reason for the relatively low yield in this step which requires improvements in further studies to come.

Enzymatic tests

The AM-ester of mannose protected with butyryl groups (**10**) was used as the model compound. First, a standard reaction was carried out without any enzyme to control the stability of the mannose phosphate under the reaction conditions. The ester was incubated in phosphate buffer at 37 °C overnight and the course of the reaction followed by TLC. Then MALDI-TOF analysis was carried out to show the stability of the ester under these conditions.

Subsequently, the ester was incubated in phosphate buffer in the presence of esterase (porcine liver, crude, EC 3.1.1.1) at 37 °C for 3 h and the reaction was controlled by TLC. Again MALDI-TOF analysis proved that the AM-groups were cleaved sucessively and that one butyryl group was removed. It may be presumed that the butyryl group in question is in position 6, as this would be the ester of a primary alcohol, and as such the most reactive site in the molecule. To our surprise, the remaining ester groups on the sugar moiety were not removed, however, as a side-product the phosphorus free mannose was detected. The same enzymatic test was conducted with pure enzyme (porcine liver, 3.2 M $(NH_4)_2SO_4$ solution, pH 8, EC 3.1.1.1). An ESI analysis was made, in addition to MALDI-TOF. It could thus be shown that again both AM-groups had been cleaved. Furthermore, the ESI spectrum demonstrated that all four butyryl protecting groups had been removed. The hydroxymethyl intermediates, produced during enzymatic cleavage, could also be detected. Further studies will be conducted in this area. Other possible substrates for the enzyme will be investigated and in vitro tests will be done.

Conclusion

In this study the preparation of various potential membrane-permeable derivatives of mannose-1-phosphate could be demonstrated combining biologically reversible phosphate and carbohydrate protecting groups. Enzymatic tests have revealed that AM-groups were cleaved to restore the original compound. It is envisaged to transfer this conception for membrane-permeant derivatives including biologically reversible protecting groups to other sugar phosphates and further biologically important components.

Experimental

General methods

NMR spectra were recorded on Bruker AC-250, AMX-400 and DRX-500. Chemical shifts of ^1H NMR and ^{13}C NMR are given relative to tetramethylsilane. Eighty-five per cent phosphoric acid was used as an external standard for ^{31}P NMR. Optical rotations were measured with Perkin–Elmer polarimeter 341. Melting points were determined with ST-apotec and are uncorrected. Elemental analyses were performed by the micro-analytical service of the Institute of Organic Chemistry of the University of Hamburg. MALDI-TOF spectra were recorded on Bruker Biflex III and ESI spectra on aHP series 1100 MSD. TLC were run on precoated plates, silica gel 60 GF_{254} (Merck). Detection was effected by observation under UV light at 254 nm, and by spraying with 10% ethanolic sulfuric acid and subsequent heating. Column chromatography was performed by flash technique using silica gel 60 (230–400 mesh, 0.040–0.063 mm, Merck).

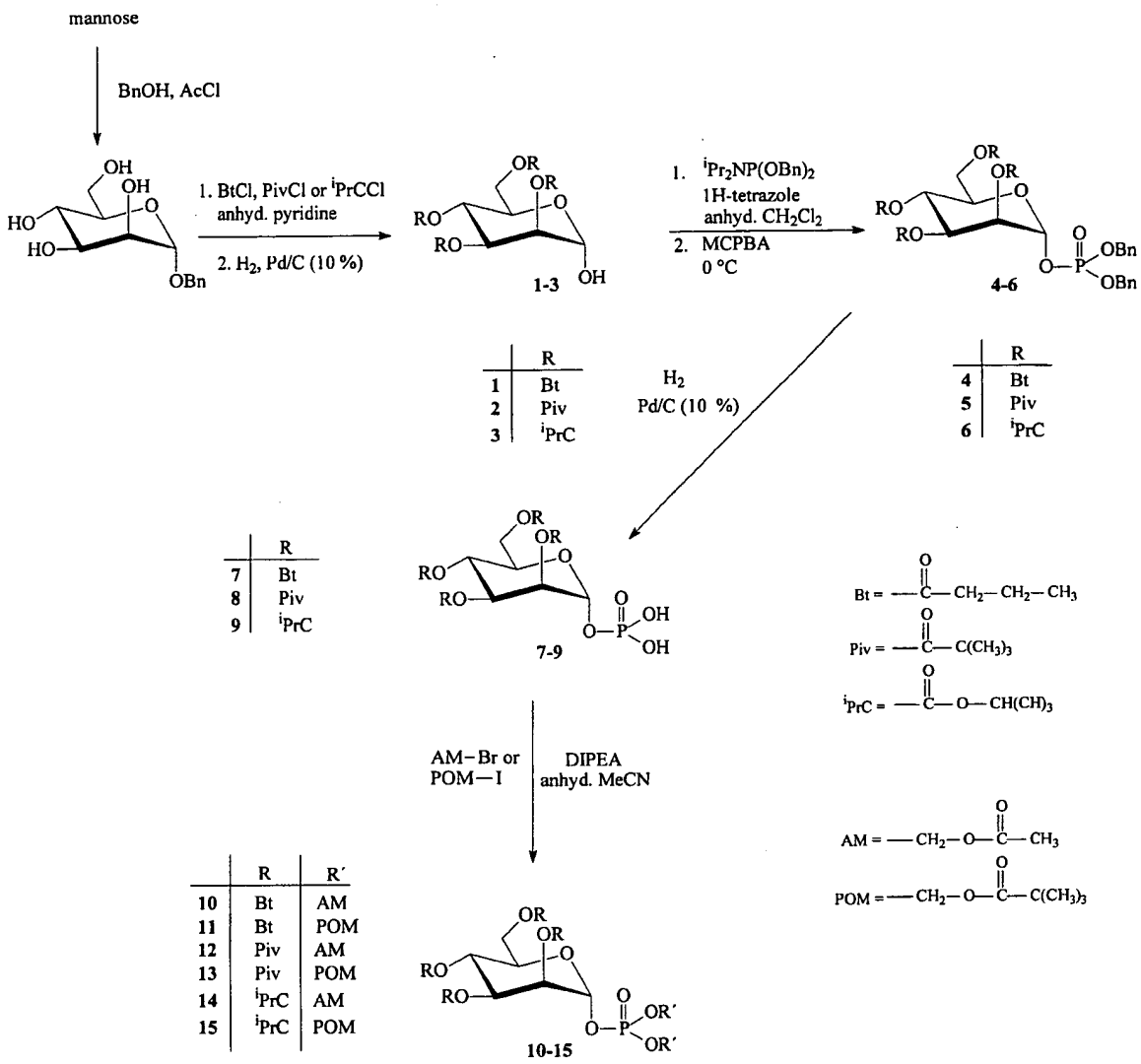
Reagents

Iodomethylpivaloate was synthesized following known procedure. Esterase (porcine liver, crude and 3.2 M $(NH_4)_2SO_4$ solution, EC 3.1.1.1) was purchased from Sigma. One unit (U) is defined as the hydrolysis of 1.0 μmol of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25 °C. DIPEA is stored over 4 Å molecular sieves.

Protection of benzyl mannopyranoside and hydrogenation[14,15]

Benzyl mannopyranoside was dissolved in dry pyridine (0.1 M solution) at 0 °C. Butyryl chloride (3 equiv/OH), pivaloyl chloride (3 equiv/OH) or *iso*-propylchlorformiate (1.5 equiv/OH, 1 M toluene) were added dropwise. The mixture was stirred overnight at room temperature.

Workup procedure for *butyryl chloride* and *pivaloyl chloride*: The reaction was quenched with methanol, then the solution concentrated and codistilled with toluene under reduced pressure. The residue was dissolved in dichloromethane, washed twice with saturated sodium



	R
1	Bt
2	Piv
3	iPrC

	R
4	Bt
5	Piv
6	iPrC

	R
7	Bt
8	Piv
9	iPrC

	R	R′
10	Bt	AM
11	Bt	POM
12	Piv	AM
13	Piv	POM
14	iPrC	AM
15	iPrC	POM

Scheme 2.

hydrogen carbonate and once with water, then dried over magnesium sulfate, filtrated, concentrated under reduced pressure and again codistilled with toluene. The crude residue was purified by column chromatography with petroleum ether/ethyl acetate (1:1).

Workup procedure for *iso-propyl chloroformiate*: The mixture was diluted with chloroform, washed twice with 1 M hydrochloric acid and once with water, then dried over magnesium sulfate, filtrated, concentrated under reduced pressure and codistilled with toluene. The crude residue was purified by column chromatography with petroleum ether/ethyl acetate (1:1). Subsequently, hydrogenation was in dry methanol (0.1 M solution) and Pd/C (10%) added cautiously and the mixture stirred at room temperature under normal H_2 pressure. After termination the solution was filtrated over Celite, concentrated under reduced pressure and purified by column chromatography with petroleum ether/ethyl acetate (3:1) to give compounds **1**, **2** or **3**.

2,3,4,6-Tetra-*O*-butyryl-α-D-mannopyranose (1). Compound **1** was synthesized in the manner described above. Yield: 0.85 g (1.85 mmol, 52% with respect to mannose, colourless sirup); R_f 0.49 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.44 (dd, 1H, H-3), 5.37 (dd∼t, 1H, H-4), 5.31 (dd, 1H, H-2), 5.24 (s, 1H, H-1), 4.27–4.16 (m, 3H, H-5, H-6a, H-6b), 3.12 (bs, 1H, OH), 2.42–2.16 (m, 8H, 4x-CO–CH_2–), 1.77–1.52 (m, 8H, 4x-CH_2–CH_3), 1.03–0.88 (m, 12H, 4x-CH_3); $J_{1,2}$ = 2.0, $J_{2,3}$ = 3.1, $J_{3,4}$ = 10.2, $J_{4,5}$ = 9.7 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 173.4, 172.7, 172.5, 172.3 (C=O), 92.4 (C-1), 69.7 (C-2), 68.8, 68.6 (C-3, C-5), 65.7 (C-4), 62.2 (C-6), 36.1, 36.0, 35.9 (–CO–CH_2–), 18.5, 18.3, 18.2 (–CH_2–CH_3), 13.7, 13.6 (–CH_3); $C_{22}H_{36}O_{10}$ (460.52).

2,3,4,6-Tetra-*O*-pivaloyl-α-D-mann pyran se (2). Compound **2** was synthesized in the manner described above. Yield: 1.38 g (2.63 mmol, 34% with respect to mannose, white crystals); mp 175.8 °C; R_f 0.23 in 3:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.53 (dd∼t, 1H, H-4), 5.46 (dd, 1H, H-3), 5.28 (dd, 1H,

H-2), 5.19 (bs, 1H, H-1), 4.29 (ddd, 1H, H-5), 4.22–4.13 (m, 2H, H-6a, H-6b), 3.17 (d, 1H, OH), 1.27, 1.24, 1.16, 1.12 (4xs, 36H, 4x-C(CH$_3$)$_3$); $J_{1,2}$ = 1.8, $J_{2,3}$ = 3.1, $J_{3,4}$ = 10.2, $J_{4,5}$ = 10.2; ^{13}C NMR (100.62 MHz, CDCl$_3$) δ 178.3, 177.3, 176.7, 172.0 (C=O), 92.5 (C-1), 69.9 (C-2), 69.1 (C-3), 68.9 (C-5), 65.2 (C-4), 61.9 (C-6), 38.9, 38.8 (C$_q$,–C(CH$_3$)$_3$), 27.2, 27.2, 27.1 (–C(CH$_3$)$_3$); $C_{26}H_{44}O_{10}$ (516.63).

2,3,4,6-Tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranose (3). Compound **3** was synthesized in the manner described above. Yield: 0.73 g (1.39 mmol, 73% with respect to mannose, colourless sirup); R_f 0.34 in 3:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, CDCl$_3$) δ 5.34 (s, 1H, H-1), 5.26–5.22 (m, 2H, H-2, H-3), 5.09 (dd∼t, 1H, H-4), 4.93–4.80 (m, 4H, 4x-CH(CH$_3$)$_2$), 4.32–4.28 (dd, 3H, H-5, H-6a, H-6b), 1.34–1.26 (m, 24H, 8x-CH(CH$_3$)$_2$); $J_{4,5}$ = 9.7 Hz; ^{13}C NMR (100.62 MHz, CDCl$_3$) δ 154.3, 153.9, 153.6, 153.4 (C=O), 92.1 (C-1), 73.0, 72.9, 72.7, 72.4 (–CH(CH$_3$)$_2$), 72.5, 72.1 (C-2, C-3), 70.0 (C-4), 68.5 (C-5), 66.1 (C-6), 21.7, 21.7, 21.6 (–CH(CH$_3$)$_2$; $C_{22}H_{36}O_{14}$ (524.52).

Phosphorylation[16]

Under argon atmosphere 1H-tetrazole (5 equiv) was suspended in dry dichloromethane (20 mL). After addition of dibenzyl di-*iso*-propylphosphoramidite (2.5 equiv) the mixture was stirred at room temperature for 15 min in order to form the tetrazolide intermediate. Then a solution of mannose derivatives **1**, **2** or **3** in dry dichloromethane (20 mL) was added and the mixture was stirred for further 3 h at room temperature before being cooled to 0 °C. MCPBA (3 equiv) was added and stirring was continued for 1 h. The solvents were removed under reduced pressure. Purification was by column chromatography with petroleum ether/ethyl acetate (3:1, 2:1) to give compounds **4**, **5** or **6**.

Dibenzyl-(2,3,4,6-tetra-*O*-butyryl-α-D-mannopyranosyl)-phosphate (4). Compound **1** (1.80 g, 3.92 mmol) was reacted in the manner described above. Yield: 2.51 g (3.48 mmol, 89%, sirup); $[\alpha]_D$ +13.7 (c 0.4, CHCl$_3$); R_f 0.45 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.40–7.30 (m, 10H, Ph), 5.62 (dd, 1H, H-1), 5.36 (dd∼t, 1H, H-4), 5.31 (dd, 1H, H-3), 5.26 (dd∼t, 1H, H-2), 5.12–5.09 (m, 4H, 2x-CH$_2$–Ph), 4.14 (dd, 1H, H-6a), 4.03 (ddd, 1H, H-5), 3.95 (dd, 1H, H-6b), 2.40–2.19 (m, 8H, 4x-CO–CH$_2$–), 1.75–1.53 (m, 8H, 4x-CH$_2$–CH$_3$), 1.01–0.88 (m, 12H, 4x-CH$_3$); $J_{1,2}$ = 1.5, $J_{2,3}$ = 3.1, $J_{3,4}$ = 10.2, $J_{4,5}$ = 9.7, $J_{5,6a}$ = 4.1, $J_{5,6b}$ = 2.0, $J_{6,6}$ = 12.2, $J_{H\text{-}1,P}$ = 6.1 Hz; ^{13}C NMR (100.62 MHz, CDCl$_3$) δ 173.1, 172.3, 172.1, 172.0 (C=O), 130.2, 129.8 (C$_q$), 128.8–128.0 (C$_{arom.}$), 95.3 (d, C-1), 70.5 (C-5), 70.0 (d,–CH$_2$–Ph), 69.9 (d,–CH$_2$–Ph), 68.6 (d, C-2), 68.2 (C-3), 64.8 (C-4), 61.4 (C-6), 36.0, 35.9, 35.8 (–CO–CH$_2$–), 18.4, 18.3, 18.2, 18.1 (–CH$_2$–CH$_3$), 13.7, 13.6 (–CH$_3$); $^2J_{C\text{-}1,P}$ = 4.8, 2x $^2J_{CH2,P}$ = 6.1, $^3J_{C\text{-}2,P}$ = 10.9 Hz; ^{31}P NMR (101.26 MHz, CDCl$_3$) δ −1.97. Anal. calcd for $C_{36}H_{49}O_{13}P$ (720.76): C 59.99, H 6.85; Found: C 60.01, H 6.74.

Dibenzyl-(2,3,4,6-tetra-*O*-pivaloyl-α-D-mannopyranosyl)-phosphate (5). Compound **2** (1.38 g, 2.63 mmol) was reacted in the manner described above. Yield: 1.43 g (1.84 mmol, 70%, sirup); $[\alpha]_D$ +22.1 (c 0.7, CHCl$_3$); R_f 0.52 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.38–7.33 (m, 10H, Ph), 5.58 (dd, 1H, H-1), 5.52 (dd∼t, 1H, H-4), 5.31 (dd, 1H, H-3), 5.24 (dd∼t, 1H, H-2), 5.16–5.08 (m, 4H, 2x-CH$_2$–Ph), 4.07–3.99 (m, 2H, H-5, H-6a), 3.89 (dd, 1H, H-6b), 1.25, 1.21, 1.14, 1.12 (4xs, 36H, 4x-C(CH$_3$)$_3$); $J_{1,2}$ = 1.9, $J_{2,3}$ = 3.2, $J_{3,4}$ = 10.4, $J_{4,5}$ = 10.1, $J_{5,6a}$ = 2.8, $J_{5,6b}$ = 1.3, $J_{6,6}$ = 12.6, $J_{H\text{-}1,P}$ = 6.3 Hz; ^{13}C NMR (100.62 MHz, CDCl$_3$) δ 178.0, 176.6, 176.4, 172.0 (C=O), 133.7–127.5 (C$_{arom.}$), 95.6 (d, C-1), 70.6 (C-5), 70.1 (d,–CH$_2$–Ph), 69.9 (d,–CH$_2$–Ph), 68.7 (C-3), 68.6 (d, C-2), 64.2 (C-4), 61.0 (C-6), 38.9, 38.8 (C$_q$,–C(CH$_3$)$_3$), 27.2, 27.1 (–C(CH$_3$)$_3$); $^2J_{C\text{-}1,P}$ = 5.6, 2x $^2J_{CH2,P}$ = 5.6, $^3J_{C\text{-}2,P}$ = 11.7 Hz; ^{31}P NMR (101.26 MHz, CDCl$_3$) δ −1.76; MALDI-TOF-MS: *m/z* 799.53 [M+Na]$^+$, 815.46 [M+K]$^+$. Anal. calcd for $C_{40}H_{57}O_{13}P$ (776.86): C 61.84, H 7.40; Found: C 61.11, H 7.35.

Dibenzyl-(2,3,4,6-tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranosyl)-phosphate (6). Compound **3** (1.19 g, 2.27 mmol) was reacted in the manner described above. 1.54 g (1.96 mmol, 87%, sirup); $[\alpha]_D$ +6.7 (c 0.5, CHCl$_3$); R_f 0.40 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.37–7.32 (m, 10H, Ph), 5.75 (dd, 1H, H-1), 5.23 (dd∼t, 1H, H-2), 5.14–5.07 (m, 6H, H-3, H-4, 2x-CH$_2$–Ph), 4.86 (m, 4H, 4x-CH(CH$_3$)$_2$), 4.26 (dd, 1H, H-6a), 4.18–4.11 (m, 2H, H-5, H-6b), 1.33–1.23 (m, 24H, 8x-CH(CH$_3$)$_2$); $J_{1,2}$ = 1.6, $J_{2,3}$ = 2.2, $J_{5,6a}$ = 5.7, $J_{6,6}$ = 11.7, $J_{H\text{-}1,P}$ = 6.6 Hz; ^{13}C NMR (100.62 MHz, CDCl$_3$) δ 154.3, 153.5, 153.4 (C=O), 128.7–128.1 (C$_{arom.}$), 94.9 (d, C-1), 73.3, 73.1, 72.8, 72.3 (–CH(CH$_3$)$_2$), 71.7 (C-3), 71.5 (d, C-2), 70.2 (C-5), 70.0 (d,–CH$_2$–Ph), 69.8 (d,–CH$_2$–Ph), 69.1 (C-4), 65.3 (C-6), 21.7–21.6 (–CH(CH$_3$)$_2$); $^2J_{C\text{-}1,P}$ = 5.6, 2x $^2J_{CH2,P}$ = 5.6, $^3J_{C\text{-}2,P}$ = 11.7 Hz; ^{31}P NMR (101.26 MHz, CDCl$_3$) δ −1.90; MALDI-TOF-MS: *m/z* 807.44 [M+Na]$^+$, 823.39 [M+K]$^+$. Anal. calcd for $C_{36}H_{49}O_{17}P$ (784.76): C 55.10, H 6.29; Found: C 55.23, H 6.45.

Hydrogenation

Pd/C (10%) was given cautiously to a solution of mannopyranosyl phosphate derivatives **4**, **5** or **6** in ethyl acetate/methanol/water (1:2:1). The mixture was stirred at room temperature under H_2 atmosphere (50 bar). After termination the solution was filtrated over Celite and concentrated under reduced pressure. The residue was purified by column chromatography with chloroform/methanol/water (6:3.5:0.5) to give products **7**, **8** or **9**.

2,3,4,6-Tetra-*O*-butyryl-α-D-mannopyranosyl phosphate (7). Compound **4** (2.43 g, 3.37 mmol) was reacted in the manner described above in 40 mL solvent for 5 h.

Yield: 1.35 g (2.50 mmol, 74%, yellow sirup); $[\alpha]_D$ +37.3 (c 1.0, CHCl$_3$); R_f 0.27 in 6:3.5:0.5 chloroform/methanol/water; ^{1}H NMR (400 MHz, CDCl$_3$) δ 5.60 (bs, 1H, H-1), 5.40 (dd, 1H, H-3), 5.36 (bs, 1H, H-2), 5.17 (dd∼t, 1H, H-4), 4.27–4.13 (m, 1H, H-5), 3.79–3.62 (m, 2H, H6a, H-6b), 2.41–2.24 (m, 8H, 4x-CO–CH$_2$–), 1.71–1.53 (m, 8H, 4x-CH$_2$–CH$_3$), 1.0–0.87 (m, 12H, 4x-CH$_3$); $J_{2,3}$ = 3.6, $J_{3,4}$ = 9.7, $J_{4,5}$ = 10.2 Hz; ^{13}C NMR (100.62 MHz, CDCl$_3$) δ 174.9–172.5 (C=O), 94.9

(bs, C-1), 72.2 (C-5), 69.1 (C-2), 68.5 (C-3), 65.8 (C-4), 61.6 (C-6), 36.0, 35.9 (–CO–$\underline{C}H_2$–), 18.4, 18.3, 18.1 (–$\underline{C}H_2$–CH_3), 13.5 (–CH_3); $^3J_{C\text{-}2,P}$ = 9.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −1.71; MALDI-TOF-MS: *m/z* 563.61 $[M+Na]^+$, 579.50 $[M+K]^+$, 585.49 $[M-H+Na+Na]^+$, 601.41 $[M-H+Na+K]^+$. Anal. calcd for $C_{22}H_{37}O_{13}P$ (540.50): C 48.89, H 6.90; Found: C 48.90, H 6.60.

2,3,4,6-Tetra-*O*-pivaloyl-α-D-mannopyranosyl phosphate (8). Compound **5** (1.30 g, 1.67 mmol) was reacted in the manner described above in 32 mL solvent for 6 h.

Yield: 0.82 g (1.37 mmol, 82%, white solid); $[\alpha]_D$ +27.0 (c 1.0, $CHCl_3$); mp ~245 °C decomposition; R_f 0.34 in 6:3.5:0.5 chloroform/methanol/water; ^{1}H NMR (400 MHz, methanol-d_4) δ 5.58 (dd~t, 1H, H-4), 5.48 (dd~t, 2H, H-1, H-3), 5.32 (bs, 1H, H-2), 4.48–4.15 (m, 3H, H-5, H-6a, H-6b), 1.28, 1.23, 1.15, 1.10 (4xs, 36H, 4x-C($\underline{C}H_3$)$_3$); $J_{3,4}$ = 10.4, $J_{4,5}$ = 10.1 Hz; ^{13}C NMR (100.62 MHz, methanol-d_4) δ 179.8, 179.3, 178.8 (C=O), 95.2 (bs, C-1), 71.7 (C-2), 71.4 (C-3), 70.8 (C-5), 66.5 (C-4), 62.9 (C-6), 40.3, 40.2, 40.1, 40.0 (C_q,–$\underline{C}(CH_3)_3$), 28.0, 27.9, 27.8 (–C($\underline{C}H_3$)$_3$; $^3J_{C\text{-}2,P}$ = 12.7 Hz; ^{31}P NMR (101.26 MHz, methanol-d_4) δ −1.75; MALDI-TOF-MS: *m/z* 619.42 $[M+Na]^+$, 635.35 $[M+K]^+$, 641.40 $[M-H+Na+Na]^+$, 657.33 $[M-H+Na+K]^+$, 673.29 $[M-H+K+K]^+$. Anal. calcd for $C_{26}H_{45}O_{13}P$ (596.61): C 52.34, H 7.60; Found: C 45.28, H 6.61 (Material hygroscopic).

2,3,4,6-Tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranosyl phosphate (9). Compound **6** (0.45 g, 0.57 mmol) was reacted in the manner described above in 8 mL solvent overnight.

Yield: 0.26 g (0.43 mmol, 75%, solid); $[\alpha]_D$ +23.4 (c 1.0, $CHCl_3$); mp 184.1 °C; R_f 0.30 in 6:3.5:0.5 chloroform/methanol/water; ^{1}H NMR (400 MHz, methanol-d_4) δ 5.58 (d, 1H, H-1), 5.26 (bs, 1H, H-2), 5.21 (dd, 1H, H-3), 5.12 (dd~t, 1H, H-4), 4.90–4.79 (m, 4H, 4x-$\underline{C}H(CH_3)_2$), 4.36–4.21 (m, 3H, H-5, H-6a, H-6b), 1.23–1.20 (m, 24H, 8x-CH($\underline{C}H_3$)$_2$); $J_{2,3}$ = 3.1, $J_{3,4}$ = 10.2, $J_{4,5}$ = 9.9, $J_{H\text{-}1,P}$ = 7.1 Hz; ^{13}C NMR (100.62 MHz, methanol-d_4) δ 156.2, 155.5, 155.1 (C=O), 95.1 (d, C-1), 74.4, 74.2, 73.8, (–$\underline{C}H(CH_3)_2$), 74.3 (C-3), 74.3 (d, C-2), 70.9 (C-4), 70.4 (C-5), 66.5 (C-6), 22.3, 22.2 (–CH($\underline{C}H_3$)$_2$); $^2J_{C\text{-}1,P}$ = 3.6, $^3J_{C\text{-}2,P}$ = 9.7 Hz; ^{31}P NMR (101.26 MHz, methanol-d_4) δ −1.02; MALDI-TOF-MS: *m/z* 627.35 $[M+Na]^+$, 643.29 $[M+K]^+$, 649.33 $[M-H+Na+Na]^+$, 665.28 $[M-H+Na+K]^+$. Anal. calcd for $C_{22}H_{37}O_{17}P$ (604.51): C 43.71, H 6.17; Found: C 44.32, H 6.06.

bis-Acetoxymethyl-(2,3,4,6-tetra-*O*-butyryl-α-D-mannopyranosyl)-phosphate (10). A solution of mannopyranosyl-1-phosphate **7** (131 mg, 0.24 mmol) in dry acetonitrile (3 mL) was evaporated to dryness. DIPEA (0.2 mL, 1.2 mmol) and dry acetonitrile (3 mL) were added and the solution was evaporated again and then in high vacuum. Subsequently, dry acetonitrile (3 mL), DIPEA (0.41 mL, 2.4 mmol) and bromomethylacetate (0.59 mL, 6.1 mmol) were added under argon. The mixture was stirred at room temperature overnight, the solvents evaporated and the residue purified by column chromatography with petroleum ether/ethyl acetate (1:1) to give **10** as a colourless sirup (68 mg, 0.1 mmol) in 41% yield; $[\alpha]_D$ +7.5 (c 0.5, $CHCl_3$); R_f 0.29 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.73–5.64 (m, 5H, H-1, 2x-CH_2–, AM), 5.41 (dd~t, 1H, H-4), 5.38 (dd~t, 1H, H-2), 5.35 (dd, 1H, H-3), 4.28–4.15 (m, 3H, H-5, H-6a, H-6b), 2.42–2.19 (m, 8H, 4x-CO–CH_2–), 2.17, 2.16 (2xs, 6H,–CH_3, AM), 1.75–1.52 (m, 8H, 4x-$\underline{C}H_2$–CH_3), 1.04–0.87 (m, 12H, 4x-CH_3); $J_{1,2}$ = 1.9, $J_{2,3}$ = 3.2, $J_{3,4}$ = 9.9, $J_{4,5}$ = 9.9, $J_{5,6a}$ = 3.8, $J_{5,6b}$ = 1.5, $J_{6,6}$ = 11.7, $J_{H\text{-}1,P}$ = 7.7 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 173.1, 172.2, 172.1 (C=O), 169.3, 169.2 (C=O, AM), 95.9 (d, C-1), 82.7 (dd~t,–CH_2–, AM), 70.8 (C-5), 68.3 (d, C-2), 68.1 (C-3), 64.7 (C-4), 61.4 (C-6), 35.9, 35.8 (–CO–$\underline{C}H_2$–), 20.6 (–CH_3, AM), 18.4, 18.3, 18.1 (–$\underline{C}H_2$–CH_3), 13.7, 13.6, 13.5 (–CH_3); $^2J_{C\text{-}1,P}$ = 6.1, $^2J_{CH2,P}$ = 6.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.05; MALDI-TOF-MS: *m/z* 707.29 $[M+Na]^+$, 723.19 $[M+K]^+$. Anal. calcd for $C_{28}H_{45}O_{17}P$ (684.51): C 49.12, H 6.63; Found: C 49.60, H 6.79.

bis-Pivaloyloxymethyl-(2,3,4,6-tetra-*O*-butyryl-α-D-mannopyranosyl)-phosphate (11). Mannopyranosyl-1-phosphate **7** (111 mg, 0.21 mmol) was suspended in dry acetonitrile (1 mL). DIPEA (0.11 mL, 0.62 mmol) and iodomethylpivaloate (0.15 g, 0.62 mmol) were added. The mixture was stirred at room temperature overnight, then the solvent removed and the residue dissolved in ethyl acetate. The mixture was washed twice with saturated brine, dried over sodium sulfate, filtrated and concentrated under reduced pressure. Purification of the crude was followed by column chromatography (petroleum ether/ethyl acetate + 1% Et_3N, 1:1) to give compound **11** (39 mg, 0.05 mmol, sirup) in 24% yield; $[\alpha]_D$ +4.1 (c 0.4, $CHCl_3$); R_f 0.88 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67–5.58 (m, 5H, H-1, 2x-CH_2–, POM), 5.34 (dd~t, 1H, H-4), 5.31 (bs, 1H, H-2), 5.29 (dd, 1H, H-3), 4.22–4.07 (m, 3H, H-5, H-6a, H-6b), 2.33 (dt, 2H, –CO–CH_2–), 2.27 (t, 2H, –CO–CH_2–), 2.19 (dt, 2H, –CO–CH_2–), 2.12 (dt, 2H, –CO–CH_2), 1.68–1.46 (m, 8H, 4x-$\underline{C}H_2$–CH_3), 1.18 (s, 18H, –C(CH_3)$_3$, POM), 0.96–0.81 (m, 12H, 4x-CH_3); $J_{1,2}$ = 1.5, $J_{2,3}$ = 3.1, $J_{3,4}$ = 9.2, $J_{4,5}$ = 9.7, $J_{5,6a}$ = 4.1, $J_{6,6}$ = 12.2 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 173.5, 172.6, 172.5, 172.4 (C=O), 96.4 (d, C-1), 83.0 (d, –CH_2–, POM), 82.8 (d, –CH_2–, POM), 70.7 (C-5), 68.3 (d, C-2), 68.1 (C-3), 64.7 (C-4), 61.4 (C-6), 39.1 (C_q, –$\underline{C}(CH_3)_3$, POM), 36.4, 36.2 (–CO–$\underline{C}H_2$–), 27.2 (–C($\underline{C}H_3$)$_3$, POM), 18.8, 18.7, 18.5 (–$\underline{C}H_2$–CH_3), 14.1, 14.0, 13.9 (–CH_3); $^2J_{C\text{-}1,P}$ = 6.1, 2x $^2J_{CH2,P}$ = 6.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −4.92; MALDI-TOF-MS: *m/z* 791.32 $[M+Na]^+$, 807.29 $[M+K]^+$. Anal. calcd for $C_{34}H_{57}O_{17}P$ (768.81): C 53.12, H 7.47; Found: C 53.75, H 7.56.

bis-Acetoxymethyl-(2,3,4,6-tetra-*O*-pivaloyl-α-D-mannopyranosyl)-phosphate (12). Mannopyranosyl-1-phosphate **8** (269 mg, 0.45 mmol) was suspended in dry acetonitrile (3 mL) and dry toluene (0.5 mL). DIPEA (0.22 mL, 1.35 mmol) and bromomethylacetae (0.13

mL, 1.35 mmol) were added. The mixture was stirred overnight at room temperature, the reaction being monitored by TLC (petroleum ether/ethyl acetate, 1:1). After further addition of bromomethylacetate (0.1 mL, 1.02 mmol) and DIPEA (0.1 mL, 0.59 mmol) the suspension was stirred again at room temperature for 2 days. The solvents were removed, the residue dissolved in ethyl acetate (5 mL) and dichloromethane (5 mL). The mixture was washed twice with saturated brine, dried over sodium sulfate, filtrated and concentrated under reduced pressure. The crude residue was purified by column chromatography (petroleum ether/ethyl acetate, 2:1) to yield **12** (104 mg, 0.14 mmol) as yellowish solid in 31%; $[\alpha]_D$ +12.0 (c 0.5, $CHCl_3$); mp 88.5 °C; R_f 0.31 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74–5.63 (m, 5H, H-1, 2x-CH_2–, AM), 5.58 (dd∼t, 1H, H-4), 5.39–5.35 (m, 2H, H-2, H-3), 4.29–4.16 (m, 3H, H-5, H-6a, H-6b), 2.17, 2.16 (2xs, 6H, –CH_3, AM), 1.28, 1.24, 11.16, 1.12 (4xs, 36H, 4x-C(CH_3)$_3$); $J_{1,2}$ = 1.8, $J_{3,4}$ = 9.4, $J_{4,5}$ = 10.2 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 178.0, 176.4 (C=O), 171.2, 169.2, 169.1 (C=O, AM), 96.2 (d, C-1), 82.7 (d,–CH_2–, AM), 82.6 (d, –CH_2–, AM), 71.0 (C-5), 68.6 (C-3), 68.4 (d, C-2), 64.1 (C-4), 61.1 (C-6), 38.9, 38.8, 38.7 (C_q, –C(CH_3)$_3$), 27.1, 27.0 (–C(CH_3)$_3$), 21.1, 20.6, 20.5 (–CH_3, AM); $^2J_{C\text{-}1,P}$ = 5.1, 2x $^2J_{CH2,P}$ = 5.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.47; MALDI-TOF-MS: *m/z* 763.52 $[M+Na]^+$, 779.46 $[M+K]^+$. Anal. calcd for $C_{32}H_{53}O_{17}P$ (740.74): C 51.89, H 7.21; Found: C 51.19, H 7.32.

bis-Pivaloyloxymethyl-(2,3,4,6-tetra-*O*-pivaloyl-α-D-mannopyranosyl)-phosphate (13). Compound **8** (232 mg, 0.39 mmol) was treated by the same procedure [dry acetonitrile (3 mL), dry toluene (1 mL), iodomethylpivaloate (0.57 g, 2.34 mmol), DIPEA (0.40 mL, 2.34 mmol), stirred for 3d] as for compound **11**. The obtained residue was purified by column chromatography (petroleum ether/ethyl acetate + 1% Et_3N, 3:1) to give **13** as a white solid (18 mg, 0.02 mmol) in 6% yield; $[\alpha]_D$ +6.1 (c 0.5, $CHCl_3$); mp 86.7 °C; R_f 0.62 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.75–5.64 (m, 5H, H-1, 2x-CH_2–POM), 5.58 (dd∼t, 1H, H-4), 5.41–5.36 (m, 2H, H-2, H-3), 4.32–4.22 (m, 2H, H-5, H-6a), 4.13 (d, 1H, H-6b), 1.27, 1.23, 1.16, 1.11 (4xs, 36H, 4x-C(CH_3)$_3$), 1.24 (2xs, 18H, 2x-C(CH_3)$_3$, POM); $J_{1,2}$ = 1.5, $J_{2,3}$ = 3.1, $J_{3,4}$ = 9.9, $J_{4,5}$ = 9.9, $J_{5,6a}$ = 2.8, $J_{6,6}$ = 11.2 $^3J_{H\text{-}1,P}$ = 5.6 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 177.9, 176.9, 176.7, 176.5 (C=O), 96.2 (d, C-1), 83.0 (d, –CH_2–, POM), 82.9 (d, –CH_2–, POM), 70.9 (C-5), 68.5 (d, C-2), 68.5 (C-3), 64.2 (C-4), 61.2 (C-6), 38.9, 38.8, 38.7 (C_q, –C(CH_3)$_3$, Piv, POM), 27.1, 27.0 (–C(CH_3)$_3$), 26.8 (–C(CH_3)$_3$, POM); $^3J_{C\text{-}1,P}$ = 5.6, 2x $^2J_{CH2,P}$ = 5.1 $^3J_{C\text{-}2,P}$ = 12.7 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.38; MALDI-TOF-MS: *m/z* 847.33 $[M+Na]^+$, 863.30 $[M+K]^+$. Anal. calcd for $C_{38}H_{65}O_{17}P$ (824.90): C 55.33, H 7.94; Found: C 56.10, H 7.99.

bis-Acetoxymethyl-(2,3,4,6-tetra-*O*-*iso*-propylcarb nate-α-D-mannopyranosyl)-phosphate (14). Mannopyranosyl-1-phosphate **9** (25.6 mg, 0.04 mmol) was treated by the same procedure [dry acetonitrile (1 mL + 1 mL + 0.5 mL), DIPEA (0.02 mL, 0.12 mmol + 0.04 mL, 0.24 mmol), bromomethylacetate (96 μL, 0.98 mmol), stirred for 3d, column chromatography with petroleum ether/ethyl acetate (1:1)] as compound **10**. Product **14** (5.4 mg, 7.2 μmol) was obtained as a colourless sirup in 17% yield.%; $[\alpha]_D$ −1.6 (c 0.6, $CHCl_3$); R_f 0.22 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 (dd, 1H, H-1), 5.73–5.64 (m, 4H, 2x-CH_2–, AM), 5.51 (dd∼t, 1H, H-2), 5.16–5.13 (m, 2H, H-3, H-4), 4.92–4.81 (m, 4H, 4x-$CH(CH_3)_2$), 4.33 (dd, 1H, H-6a), 4.28–4.22 (m, 2H, H-5, H-6b), 2.18, 2.16 (2xs, 6H, –CH_3, AM), 1.34–1.25 (m, 24H, 4x-$CH(CH_3)_2$); $J_{1,2}$ = 1.5, $J_{2,3}$ = 2.0, $J_{5,6a}$ = 6.4, $J_{6,6}$ = 12.2 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 168.3, 168.2 (C=O, AM), 154.2, 153.5, 153.4, 153.3 (C=O), 95.5 (d, C-1), 82.8 (d, –CH_2–, AM), 82.7 (d,–CH_2–, AM), 73.5, 73.2, 72.9, 72.4 (–$CH(CH_3)_2$), 71.5, 68.9 (C-3, C-4), 71.2 (d, C-2), 70.5 (C-5), 65.2 (C-6), 21.7, 21.6, 21.5 (–$CH(CH_3)_2$), 20.6, 20.5 (–CH_3, AM); $^2J_{C\text{-}1,P}$ = 5.1, 2x $^2J_{CH2,P}$ = 5.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.30; MALDI-TOF-MS: *m/z* 771.08 $[M+Na]^+$, 787.03 $[M+K]^+$. Anal. calcd for $C_{28}H_{45}O_{21}P$ (748.51): C 44.92, H 6.06; Found: C 45.09, H 6.20.

bis-Pivaloyloxy-(2,3,4,6-tetra-*O*-*iso*-propylcarbonate-α-D-mannopyranosyl)-phosphate (15). Compound **9** (189 mg, 0.31 mmol) was suspended in dry acetonitrile (3 mL). DIPEA (0.16 mL, 0.94 mmol) and iodomethylpivaloate (0.23 g, 0.94 mmol) were added. The mixture was stirred overnight at room temperature and the course of the reaction followed by TLC (petroleum ether/ethyl acetate, 1:1). The mixture was stirred for a further 3 days after addition of DIPEA (0.16 mL) and iodomethylpivaloate (0.23 g). The solvents were removed, the residue was dissolved in ethyl acetate (5 mL) and dichloromethane (5 mL). The mixture was washed twice with saturated brine, dried over sodium sulfate, filtrated and concentrated under reduced pressure. The crude residue was purified by column chromatography (petroleum ether/ethyl acetate + 1% Et_3N, 3:1) to yield **15** (9.3 mg, 0.01 mmol) as colourless sirup in 4%; $[\alpha]_D$ +2.3 (c 0.5, $CHCl_3$); R_f 0.5 in 1:1 petroleum ether/ethyl acetate; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78 (dd, 1H, H-1), 5.75–5.65 (m, 4H, 2x-CH_2–, POM), 5.30 (dd∼t, 1H, H-2), 5.16–5.12 (m, 2H, H-3, H-4), 4.91–4.81 (m, 4H, 4x-$CH(CH_3)_2$), 4.33 (dd, 1H, H-6a), 4.27–4.22 (m, 2H, H-5, H-6b), 1.32–1.26 (m, 24H, 4x-$CH(CH_3)_2$), 1.24, 1.23 (2xs, 18H, 2x-C(CH_3)$_3$, POM); $J_{1,2}$ = 1.8, $J_{2,3}$ = 4.9, $J_{5,6a}$ = 6.4, $J_{5,6b}$ = 2.5, $J_{6,6}$ = 12.2, $J_{CH,CH3}$ = 6.4, $^3J_{H\text{-}1,P}$ = 5.6 Hz; ^{13}C NMR (100.62 MHz, $CDCl_3$) δ 95.5 (d, C-1), 83.0 (d, –CH_2–, POM), 82.9 (d, –CH_2–, POM), 73.4, 73.1, 72.9, 72.4 (–$CH(CH_3)_2$), 71.6 (C-3), 71.3 (d, C-2), 70.5 (C-5), 69.0 (C-4), 65.2 (C-6), 26.8 (–C(CH_3)$_3$, POM), 21.7, 21.6 (–$CH(CH_3)_2$); $^3J_{C\text{-}1,P}$ = 5.1, 2x $^2J_{CH2,P}$ = 5.1, $^3J_{C\text{-}2,P}$ = 12.2 Hz; ^{31}P NMR (101.26 MHz, $CDCl_3$) δ −5.23; MALDI-TOF-MS: *m/z* 855.26 $[M+Na]^+$, 871.22 $[M+K]^+$. Anal. calcd for $C_{34}H_{57}O_{21}P$ (8.32.79): C 49.04, H 6.90; Found: C 50.45, H 7.34.

Enzymatic tests

Crude enzyme. Compound **10** (10.1 mg) was incubated in phosphate buffer (pH 7.4, 0.05 M, 1 mL) with porcine

liver esterase (crude, EC 3.1.1., 1 U) at 37 °C for 3 h. The reaction was monitored by TLC (petroleum ether/ethyl acetate, 1:1). After termination by quenching with methanol (2 mL) the residue was centrifuged and desalted using $ZipTip_{C18}$ for direct spotting on a MALDI-TOF MS target. Methanol/water solution in various ratio was used for elution; MALDI-TOF-MS: *m/z* 706.98 $[M+Na]^+$, 722.92 $[M+K]^+$, 635.21 $[M-AM+Na]^+$, 651.19 $[M-AM+K]^+$, 657.27 $[M-AM-H+Na+Na]^+$, 673.24 $[M-AM-H+Na+K]^+$, 563.23 $[M-2AM+Na]^+$, 585.24 $[M-2AM-H+Na+Na]^+$, 601.21 $[M-2AM-H+Na+K]^+$, 587.26 $[M-AM-Bt-H+Na+Na]^+$, 515.31 $[M-2AM-Bt-H+Na+Na]^+$, 483.40 $[M-PO_3AM_2+Na]^+$/$[M-2Bt-2Ac+2H+Na]^+$, 499.31 $[M-PO_3AM_2+K]^+$/$[M-2Bt-2Ac+2H+K]^+$, 413.68 $[M-PO_3AM_2-Bt+Na]^+$, 429.63 $[M-PO_3AM_2-Bt+K]^+$.

Pure enzyme. Compound **10** (9.7 mg) was incubated in phosphate buffer (pH 7.4, 0.05 M, 1 mL) with porcine liver esterase (pure, EC 3.1.1.1, 1 U) at 37 °C for 3 h. The reaction was monitored by TLC (petroleum ether/ethyl acetate, 1:1). After termination by quenching with methanol (2 mL) the residue was centrifuged and desalted using $ZipTip_{C18}$ for direct spotting on a MALDI-TOF MS target and ESI analysis. Methanol/water solution in various ratio was used for elution; MALDI-TOF-MS: *m/z* 707.19 $[M+Na]^+$, 723.09 $[M+K]^+$, 635.22 $[M-AM+Na]^+$, 651.17 $[M-AM+K]^+$, 657.25 $[M-AM-H+Na+Na]^+$, 673.22 $[M-AM-H+Na+K]^+$, 611.19 $[M-Bt-Ac+H+K]^+$, 623.23 $[M-2Ac+H+Na]^+$, 563.21 $[M-2AM+Na]^+$, 585.22 $[M-2AM-H+Na+Na]^+$, 601.11 $[M-2AM-H+Na+K]^+$, 483.44 $[M-PO_3AM_2+Na]^+$/$[M-2Bt-2Ac+2H+Na]^+$, 499.22 $[M-PO_3AM_2+K]^+$/$[M-2Bt-2Ac+2H+K]^+$; ESI-MS: *m/z* 354 $[M-AM-4Bt+Na]^+$, 353 $[M-2AM-3Bt+Na]^+$, 283 [M–2AM–4Bt + Na].

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